EVOLUTION OF WIND POLLINATION IN *LEUCADENDRON* (PROTEACEAE): EXPERIMENTAL EVIDENCE AND FLORAL TRAIT SHIFTS

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As the candidate’s supervisor I have approved this thesis for submission.

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ABSTRACT

Evolutionary transitions from insect to wind pollination are thought to have occurred many times during the angiosperm radiation. This transition is commonly associated with a suite of distinctive floral traits such as reduction of flower size and a transition to dry pollen. In the dioecious genus, *Leucadendron* (Proteaceae), evolutionary shifts from insect to wind pollination have been postulated based on floral morphology features. In this study, I aimed to experimentally test the potential for wind versus insect pollination in several *Leucadendron* species and document a variety of floral traits (pollen size, inflorescence size, scent, colour, etc.) in order to determine their functional significance whilst utilizing phylogenetic comparative methods to test the statistical significance of evolutionary associations between particular floral traits and pollination systems.

Fifteen representative *Leucadendron* species were investigated to verify insect and wind pollination in as many clades as possible. Insect exclusion experiments confirmed that five *Leucadendron* species, *L. rubrum*, *L. salicifolium*, *L. dubium, L. coniferum* and *L. teretifolium* are indeed wind-pollinated. *Pria cinerascens* (Nititulidae) was found to be the main pollinator of the insect-pollinated *Leucadendron* species due to their abundance, high stigmatic contact and relatively pure *Leucadendron* pollen loads. Overall, however, the abundance of insects visiting inflorescences was not significantly different between insect- and wind-pollinated species, which highlights the importance of conducting insect exclusion experiments to evaluate whether a species is wind- or insect-pollinated.

From the previously determined pollination systems of 17 *Leucadendron* species, floral traits associated with the shift to wind pollination were investigated to determine whether transitions from insect to wind pollination were accompanied by modifications of pollination-relevant floral traits. In a wind tunnel, pollen grains of wind-pollinated species were found to be more motile than those of insect-pollinated species. Phylogenetic analyses suggest that transitions from insect to wind pollination occurred at least four times during the diversification of *Leucadendron* and that, compared to insect-pollinated cogeners, wind-pollinated *Leucadendron* species are characterized by increased production of smaller pollen grains, higher inflorescence density, less attractive visual and olfactory cues, and a greater degree of sexual dimorphism for these visual and olfactory cues.

In conclusion, this study experimentally confirms that there were several shifts from insect to wind pollination in *Leucadendron* and identifies floral traits that were evolutionarily modified during these shifts.

PREFACE

The research described in this dissertation was carried out in the School of Life Sciences (Pietermaritzburg campus) from March 2011 to September 2014 under the supervision of Prof. Steven D. Johnson and Prof. Jeremy J. Midgley (University of Cape Town).

The work presented in this dissertation represents the original work of the author and has not been otherwise submitted in any other form for any degree or diploma to any other University. Where use has been made of the work of others, this has been duly acknowledged in the text.

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Megan R. Welsford
September 2014

I certify that the above statement is correct

JJ Midgley

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Prof. Steven D. Johnson (supervisor) Prof. Jeremy J. Midgley (co-supervisor)
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DECLARATION 1 – PLAGIARISM

I, Megan R. Welsford declare that

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS THAT FORM PART OF AND/OR INCLUDE RESEARCH PRESENTED IN THIS THESIS

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MRW, JJM and SDJ conceived paper. MRW collected and analysed data, and wrote the paper. SDJ and JJM contributed comments.

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CONTENTS

CHAPTER 1

INTRODUCTION

Understanding what led to the radiation and diversification among flowering plants has been a main objective for many botanists and evolutionary biologists. The most convincing hypothesis for this extraordinary diversification of angiosperms, initially proposed by Darwin (1859, 1862, 1877), is the role of animal pollinators, especially insects (Stebbins, 1970; Dodd et al., 1999; Vamosi and Vamosi, 2010). The evolutionary shifts between different animal pollinators is associated with modifications of floral traits and thought to be a key force in angiosperm diversification (Stebbins, 1970; Johnson, 2006; Campbell, 2008; Harder and Johnson, 2009; van der Niet and Johnson, 2012; van der Niet et al., 2014a).

The variation in animal pollinator's distribution and abundance over a plant's geographical distribution has resulted in frequent shifts between pollination systems, which can lead to speciation, since these transitions usually have implications for both reproductive isolation and divergence of the floral phenotype (Grant and Grant, 1965; Stebbins, 1970; Johnson, 2006; Campbell, 2008; van der Niet and Johnson, 2012). According to Grant and Grant (1965) and Stebbins (1970, 1973, 1981), who developed the concepts for understanding pollinator-shifts, the vast variety of floral traits present in flowering plants is due to adaptations to different pollinators with varied sensory capabilities, behaviours and morphologies. However, much is still unknown about what floral trait modifications are important to bring about pollinator shifts (Schemske and Bradshaw, 1999; Shuttleworth and Johnson, 2010) and whether pollination systems sharing few or numerous traits in common have a greater likelihood for shifts to take place (Aigner, 2001). To expand our understanding of floral trait evolution related to pollinator shifts, not only should the most efficient pollinators be identified and established (Grant and Grant, 1965; Stebbins, 1970), but also the advertising traits and floral rewards of plant species, and all of this should be incorporated into the framework of a resolved phylogeny (van der Niet and Johnson, 2012).

Recent studies have added to our knowledge of floral traits associated with evolutionary shifts from one animal pollinator to another, such as from moth to long-proboscid fly (Johnson et al., 2002), bee to hummingbird (Kay et al., 2005; Wilson et al., 2007; Thomson and Wilson, 2008), from bee to bird to hawkmoth (Cosacov et al., 2009), from bee to beetle pollination (Peter and Johnson, 2014), from bird to moth pollination (van der Niet et al., 2014b) and from bird to butterfly pollination (Kiepel and Johnson, 2014). Furthermore, a spate of recent studies has shown how variations in floral scent can play a vital role in pollinator shifts (Muchhala and Thomson, 2010; Peakall et al., 2010;

Shuttleworth and Johnson, 2010; Wragg and Johnson, 2011; Peakall and Whitehead, 2014; Peter and Johnson, 2014; Sun et al., 2014; Van der Niet et al., 2014b)

However, if alterations to the abiotic or biotic environment result in pollinators to be absent, limited or that they deliver poor quality pollen, a possible outcome could be a shift to wind pollination (Whitehead, 1969; Regal, 1982; Niklas, 1985; Berry and Calvo, 1989; Cox, 1991; Weller et al., 1998; Goodwillie, 1999) or the evolution of self-fertilization (Lloyd, 1979; Schoen et al., 1996; Kalisz et al., 1999). For instance, in *Espeletia* (Asteraceae) occurring in the Venezuelan Andes, shifts from bird and insect pollination to wind pollination are associated with a decline of animal pollinators with increased altitude (Berry and Calvo, 1989). In such circumstances, plants might incorporate a combination of both wind and animal pollination (ambophily), either simultaneously or successively (see review by Culley et al., 2002). However, it is unknown whether ambophily is an evolutionary stable strategy due to its plasticity or just an intermediate stage in the shift to full wind pollination (Culley et al., 2002; Friedman and Barrett, 2009)

SHIFTS FROM ANIMAL TO WIND POLLINATION

The evolutionary shift from animal pollination to wind pollination signifies one of the major transitions in flowering plants (Culley et al., 2002; Friedman and Barrett, 2009). Wind pollination has evolved frequently in numerous flowering plant lineages, arising at least 65 times (Faegri and van der Pijl, 1979; Linder, 1998) and is presently found in approximately 18% of families (Ackerman, 2000) such as Poaceae, Cyperaceae, Juncaceae, and Betulaceae (Proctor and Yeo, 1973; Faegri and van der Pijl, 1979; Culley et al., 2002).

Wind pollination is often considered a fairly passive process (compared to animal pollination); for successful pollen dispersal and pollination to take place not only is wind vital but also other abiotic factors, such as low rainfall and low humidity (Culley et al., 2002). Ecological circumstances favouring the evolution of wind pollination include dry and open environments that assist the aerodynamic requirements of pollen dispersal (Whitehead, 1969; Niklas, 1985; Weller et al., 1998; Ackerman, 2000; Culley et al., 2002) and densely populated conspecific plants (Pannell and Barrett, 1998; Davis et al., 2004).

Regionally and geographically, wind-pollinated species usually occur in higher elevations and latitudes, especially in temperate forests and grasslands, but rarely in the tropical lowland rainforests (Regal, 1982; Whitehead, 1983; Culley et al., 2002; Chazdon et al., 2003). A recent study by Rosa and Koptur (2013), however, found that pollen dispersal by wind was just as effective in the forest habitats as in the savanna habitats for a wind-pollinated Amazonia palm species, *Mauritia flexuosa* L.f., suggesting that differences in habitats might not greatly impact the pollen transportation as previously thought.

Wind pollination mainly occurs by the collision of pollen grains with the stigmas as the air current carrying the pollen grains passes by the stigma (Niklas, 1985). Therefore, there is an undeniable importance of the airflow aerodynamics associated with plant architecture for both pollen release and capture (Niklas, 1985, 1987). Indeed, wind pollination is more likely to evolve in taxa with the presence of suitable plant architecture that realizes these aerodynamic requirements (Bickel and Freeman, 1993). Therefore, the position of inflorescences in the airstream (Niklas, 1987) and changes in inflorescence structure can confer greater ability for pollen dispersal or deposition (Niklas and Buchmann, 1985; Niklas, 1987). In *Festuca campestris* Rydb., florets held in upper positions captured greater amounts of pollen grains because high volumes of air sweeps through them during oscillation (Friedman and Harder, 2004; Friedman and Barrett, 2009). Niklas and Buchmann's (1985) wind tunnel study on *Simmondsia chinensis* C.K.Schneid. found that the position and angle of its leaves in the air stream could deflect airborne pollen onto flowers and that the flowers could deflect pollen downwards and upwards to other flowers. In the wind-pollinated species, *Schiedea globosa* H.Mann, changes to the inflorescence (containing unisexual flowers) included it being greatly condensed and subtended by an elongated peduncle with small leaves for pollen release and capture (Weller et al., 1998).

Apart from plant and inflorescence structure, the shift to wind pollination is frequently associated with a specific suite of floral morphological traits, including reduced or absent perianth, small flowers with less (often single) ovules and loss of nectaries (Faegri and van der Pijl, 1979; Culley et al., 2002; Friedman and Barrett, 2008, 2009; Wragg and Johnson, 2011). For example, in comparison to the insect-pollinated *Acer pseudoplatanus* L., the wind-pollinated species *Acer negundo* L. lacks both nectaries and petals, and has a larger stigma (Soltis et al., 2005). The pollen grains of wind-pollinated species generally tend to be smoother, drier, lighter and more consistently sized (17-58um) than those of animal-pollinated species, to possibly aid in dispersal by wind (Friedman and Barrett, 2008, 2009; Wragg and Johnson, 2011). Rosa and Koptur (2013) noted several morphological traits made the experimentally proven wind-pollinated palm species, *M.*

flexuosa, adapt for wind pollination, such as immense pollen production, numerous flowers, unisexual flowers, absent nectaries and reduced ovules per flower. However, only a few studies have considered the floral traits of floral colouration and scent in wind-pollinated species (Magalhães et al., 2005; Wragg and Johnson, 2011). A study by Wragg and Johnson (2011) found floral scent and colour were important functional traits in the shift from wind pollination to insect pollination in sedges. Overall, however, phylogenetic analyses suggest that just a few trait alterations are required for the transition to wind pollination, in particular dry pollen and small flowers (Linder, 1998; Wragg and Johnson, 2011).

Evolutionary shifts from animal to wind pollination may be evident in the dioecious genus *Leucadendron* (Proteaceae). Williams (1972) speculated that several *Leucadendron* species might be wind-pollinated, based mainly on floral trait shifts. This thesis seeks to addresses the claim of the evolutionary shift to wind pollination within the genus, and to identify floral trait modifications that may be associated with such shifts.

DIOECY AND SEXUAL DIMORPHISM IN WIND-POLLINATED SPECIES

A common feature among wind-pollinated species is the high incidence of dioecy (Bawa, 1980; Charlesworth, 1993; Renner and Ricklefs, 1995; Vamosi et al., 2003; Friedman and Barrett, 2008). Indeed, according to Renner and Ricklefs (1995), an estimated 30% of genera containing dioecious species are wind-pollinated. A possible explanation for this trend is that dioecy promotes outcrossing and consequently avoids potentially detrimental inbreeding depression caused by self-pollination (Renner and Ricklefs, 1995; Culley et al., 2002; Friedman and Barrett, 2009).

The evolution of wind pollination has been suggested to develop either before (Charlesworth, 1993; Renner and Ricklefs, 1995) or after (Kaplan and Mulcahy, 1971) dioecy has evolved. In the genus *Acer* (Aceraceae), where wind pollination is suggested to evolve before dioecy, only *A. negundo* has evolved complete wind pollination and also full dioecy (de Jong, 1976). Dioecy appears to have evolved after the evolution of wind pollination in the lower rosids (Linder, 1998), but concurrent evolution is also apparent in some cases. If dioecy evolves prior to wind pollination, animal pollinators might not visit female plants because they lack pollen rewards, which could result in a decrease in female fecundity (Charlesworth, 1993). For example, in *Thalictrum* (Ranunculaceae) which has both insect- and wind-pollinated species, there is evidence that female flowers are discriminated against by insect pollinators because they lack pollen (see Kaplan and

Mulcahy, 1971) However, pollen-seeking pollinators of dioecious species can be effectively deceived (Culley et al., 2002). For instance, they could be attracted to a female flower with no rewards because the female plants mimic pollen producing male flowers (Castillo et al., 1999).

Sexual dimorphism among dioecious species tends to be more pronounced in wind-pollinated species than in animal-pollinated species (Lloyd and Webb, 1977; Weller et al., 2007; Harris and Pannell, 2010). In *Schiedea* (Caryophyllaceae), an endemic Hawaiian genus, all strongly sexually dimorphic species appear to be wind-pollinated (Weller et al., 1998). The main suggestion for this trend is that while animal-pollinated species have to place their stigmas and anthers in similar localities to ensure reliable pollinator behaviour for successful pollen transfer (Lloyd and Webb, 1977; de Jong and Klinkhamer, 2005), wind-pollinated species lack this constraint (Lloyd and Webb, 1986; Vamosi and Otto, 2002; Weller et al., 2007). Furthermore, wind-pollinated dioecious plants do not have to have similar visual advertising signals for male and female flowers. In wind-pollinated species, the architectural and morphological divergence should arise because male plants should select for more effective pollen dispersal, while female plants should select for more efficient pollen receipt (Weller et al., 2007; Friedman and Barrett, 2009). Indeed, the considerable inflorescence and/or architectural dimorphism in wind-pollinated species, such as *Leucadendron rubrum* Burm.f. (Friedman and Barrett, 2009) and *Buchloe dactyloides* Engelm. (Quinn, 1991), could be as a result of different optimal selection for pollen dispersal and receipt (Weller et al., 2007).

RELATIVE ADVANTAGE OF WIND POLLINATION

In comparison to animal pollination, wind pollination (anemophily) has often been considered to be a wasteful, random and inefficient process, mostly due to excessive pollen production and the possible unpredictability of environmental conditions (Faegri and Van der Pijl, 1979; Niklas, 1985; Ackerman, 2000; Hall and Walter, 2011). Indeed, even Charles Darwin was puzzled by this phenomenon:

> “As a large quantity of pollen is wasted by anemophilous plants, it is surprising that so many vigorous species of this kind abounding with individuals should still exist in any part of the world; for if they had been rendered entomophilous, their pollen would have been transported by the aid of the senses and appetites of insects with incomparably greater

> safety than by the wind... It seems at first sight a still more surprising fact that plants, after having been once rendered entomophilous, should ever have again become anemophilous”.
>
> (Darwin 1876, p. 407)

However, recent experimental evidence has proven that this previous notion about wind-pollinated species having wasteful pollen dispersal is unfounded (see Friedman and Barrett, 2009). Furthermore, Midgley and Bond (1991) suggested that under some circumstances wind pollination can be an extremely effective and a viable alternative to animal pollination. Why and when would the evolution of wind pollination be advantageous?

A main advantage of wind pollination is the potential of providing species with reproductive assurance. Since unreliable and possible erratic pollinators are commonly thought of as the selective force favouring the evolution of wind pollination from animal pollination, being independent of pollinators could then provide partial or full reproductive assurance (Goodwillie, 1999; Friedman and Barrett, 2009; Hesse and Pannell, 2011). Indeed, Goodwillie (1999) found that wind pollination in *Linanthus parviflorus* Benth. (Greene) provided considerable reproductive assurance and could allow self-incompatibility to persist during periods of low pollinator visitation.

According to Cox (1991), wind pollination might be advantageous and superior in locations with particularly short growing seasons and newly inhabited ecosystems. Furthermore, high proportions of wind-pollinated plants are present in low-species diversity localities where insects are sparse to pollinate plants, like saltmarshes and semi-arid environments (Cox, 1991). Effective long-distance pollen dispersal and pollination is also evident in some wind-pollinated plants that are not hindered by animal pollinators’ range (Cox, 1991). For instance, pollen dispersal up to 40 km has been recorded for the tropical wind-pollinated tree, *Cecropia obtusifolia* Bertol. (Kaufman et al., 1998).

Wind-pollinated plants often have the advantage of being less prone to pollen limitation than their animal-pollinated counterpart (Whitehead, 1969; Weller et al., 1998; Goodwille, 1999; Culley et al., 2002). For instance, Friedman and Barrett (2009) study on wind-pollinated herbs, both dioecious and monoecious species, determined that adding supplementary pollen on stigmas did not yield greater seed set, and that similar pollen loads were recorded on stigmas of wind- and animal-pollinated species, indicating the effectiveness of outcrossing in wind-pollinated species. However, this effectiveness of outcrossing often relies upon the pollen donor’s proximity and therefore population density

(Rognli et al., 2000; Stehlik et al., 2006; Steven and Waller, 2007; Hess and Pannell, 2011). Consequently, densely packed plant populations are the most effective for successful wind pollination and pollen limitation can occur at low population densities where mates are too far apart (Pannell and Barrett, 1998; Knapp et al., 2001; Davis et al., 2004; Hess and Pannell, 2011). Certainly one of the reasons for the relatively low frequency of wind-pollinated species occurring in rain forests is due to mainly low plant population densities occurring in rain forests (Regal, 1982).

Finally, wind-pollinated species could escape being dependent on pollinators whose distribution could be climatically limited and susceptible to extinction (Cox, 1991). Indeed, whereas, the majority of endangered plants in the western United States of America are insect-pollinated, only a few are wind-pollinated (Harper, 1979). Therefore, Cox (1991) suggested that in wind-pollinated species the rate of extinction should probably be lower than in animal-pollinated species.

THE STUDY SYSTEM AND AIMS

Leucadendron R. Br., forming part of the ancient Proteaceae family, is comprised of ~ 96 taxa (85 species and 11 subspecies) species (Williams, 1972; Barker et al., 2004) which are near-endemic to the Cape Floristic Region (CFR) of South Africa (Williams, 1972). Moreover, *Leucadendron*, together with other genera from the Leucadendreae tribe, probably originated in the CFR due to long-distance dispersal from ancestors in Australia (see Sauquet et al., 2009). It is one of only four other dioecious genera in the Proteaceae (the others being *Aulax*, *Dilobeia* and *Heliciopsis*) (Barker et al., 2004). Female plants have cone-like inflorescence that becomes woody after pollination by wind or insect (Williams, 1972; Collins and Rebelo, 1987; Midgley, 1987; Hattingh and Giliomee, 1989). For numerous species, the cones become serotinous, remaining on the female plant where seeds accumulate and after fire germination occurs, following the death of adult plants and the release of seeds (Bond and Maze, 1999). This is considered an essential adaptation in the fire prone, nutrient poor soil environment of the fynbos (Cowling and Holmes, 1992; Barker et al., 2004).

The genus is divided into two main sections based on seed morphology: (1) *Leucadendron* (fruits are flat) and (2) *Alatospermum* (fruits are round nuts) (Williams, 1972). These two sections were further divided into 14 subsections based on seed morphology, habitat, cone size, morphology leaf and basal stem being singular or multiple (Williams, 1972). However, a molecular phylogenetic study by Barker et al. (2004), using

ITS sequence data, did not support the sectional and sub-sectional divisions. They found that the *Leucadendron* section was paraphyletic and the subsections were not constantly monophyletic.

Leucadendron species are considered to be either insect- or wind-pollinated, with an estimated 6.6% being wind-pollinated and 89% being insect-pollinated (Williams, 1972). The remaining species are either extinct (3.3%) or possibly in transition between insect and wind pollination (1.1%; Williams, 1972). Wind pollination was inferred by Williams (1972) for six *Leucadendron* species based on floral trait shifts including no distinctive floral odour, lack of conspicuous coloured involucral leaves, reduced stigmatic surface and showering of pollen when branches are shaken. In a phylogenetic reconstruction of shifts using floral syndromes, Barker et al. (2004) concluded wind pollination has evolved independently several times in the genus and that insect pollination was the ancestral state within the genus.

Among insect-pollinated *Leucadendron* species, beetles (Coleoptera) appear to be the main pollinators (Hattingh and Giliomee, 1989; Hemborg and Bond, 2005). The beetle commonly associated with the inflorescences of *Leucadendron* is a tiny pollen beetle, *Pria cinerascens* Er. (Nitidulidae) and is particularly common on the male inflorescences, where they live their lives feeding on pollen and breed (Williams, 1972; Hattingh and Giliomee, 1989; Hemborg and Bond, 2005). In their pollination study, Hattingh and Giliomee (1989) determined *P. cinerascens* as the pollinator of several *Leucadendron* species including *L. laureolum* (Lam.) Fourc., *L. salignum* P.J. Bergius and *L. microcephalum* (Gand.) Gand. & Schinz. Furthermore, *P. cinerascens* was the main pollinator of *L. xanthoconus* (Kuntze) K. Schum. (Hemborg and Bond, 2005). Nitidulids play an important role in pollination of several other plant families for instance Arecacae (Listabarth, 1996), Brassicaceae (Hopkins and Ekom, 1999) and Annonaceae (Gottsberger, 1999).

Numerous *Leucadendron* species show varying degrees of sexual dimorphism (Williams, 1972). Several studies have investigated sexual dimorphism within *Leucadendron* (Bond and Midgley, 1988; Bond and Maze, 1999; Hemborg and Bond, 2005; Harris and Pannell, 2010; Midgley, 2010). Among these studies, the main focus has been the differences in leaf size between the sexes (Bond and Midgley, 1988; Midgley, 2010). Midgley (2010) suggested that among flowering plants, the genus might have the highest degree of gender-linked leaf dimorphism. Overall, wind-pollinated *Leucadendron* species tend to have a greater degree of leaf size dimorphism than insect-pollinated species

(Bond and Midgley, 1988), possibly given that the architectural structure requirements to disperse and receive wind-borne pollen differ so noticeably (Lloyd and Webb, 1977, 1986; Friedman and Harder, 2004). Harris and Pannell (2010) found that the degree of sexual dimorphism was more pronounced in serotinous species, with females being selected to be less branched than males to maintain the costs of producing cones. Bond and Midgley (1988) noted *Leucadendron* species with large yellow conspicuous involucral leaves showed less degrees of sexual dimorphism in leaf size and inflorescence number. They, therefore, suggested that sexual dimorphism in relation to vegetative traits should be greater in dioecious species where effective pollination relies on the quantity of pollen produced and not the quality of floral rewards and attractants. However, sexual dimorphism in *L. xanthoconus,* an insect-pollinated species, is suggested to be as a result of its nitidulid beetle pollinator, *P. cinerascens*, obtaining different rewards in male (pollen) and female (shelter) inflorescences (Hemborg and Bond, 2005).

Overall the pollinators of *Leucadendron* species appear to be efficient, given that fruit and seed set in *Leucadendron* species are relatively high, with Rebelo and Rourke (1986) claiming overall fruit set for 87 species to be as high as 77%. This yields the interesting question as to why, given the effectiveness of pollinators, wind pollination would evolve providing that wind pollination is often associated with pollinators become less effective and/or pollen limitation (Whitehead, 1969; Niklas, 1985; Cox, 1991; Goodwillie, 1999; Ackerman, 2000; Hall and Walter, 2011). Firstly, dioecy in the genus could be linked to this high reproductive output, since it lends to outcrossing (Baker, 1984) and especially given that in most hermaphroditic Proteaceae fruit and seed set is low (9.2 % and 5.6% fruit set for *Protea* and *Leucaspermum*, respectively) (see Collins and Rebelo, 1987). Furthermore, wind pollination might possibly be equally as or more efficient in the genus. For example, Linder and Midgley (1996) found that 80.2% of the pollen on the stigmas of *Leucadendron rubrum* Burm. f. (presumed wind-pollinated species) was that of *Leucadendron* and probably of its own; and Hattingh and Giliomee (1989) found that exclusion of insects carrying pollen but not airborne pollen had little effect on seed set in *Leucadendron coniferum* (L.) Meisn. To our knowledge, Hattingh and Giliomee’s (1989) study is the only previous experimental evaluation of wind pollination in a *Leucadendron* species.

Previous studies on genera with apparent wind-pollinated systems such as *Salix* (Salicaceae) (Vroege and Stelleman, 1990; Peeters and Totland, 1999), *Schiedea* (Caryophyllaceae) (Norman et al., 1997), and *Linanthus* (Polemoniaceae) (Goodwillie, 1999), have shown that even though floral traits suggest that species are either wind- or insect-pollinated, both wind and insects contribute to seed production (i.e. ambophilous). This indicates the necessity to implement experimentation to confirm wind pollination, instead of relying on inferences from floral syndromes. Therefore the aims of this thesis were to 1) experimentally compare the potential for insect and wind pollination in a sample of *Leucadendron* species chosen to be representative of as many clades as possible and 2) compare floral traits and sexual dimorphism from these confirmed insect- and wind-pollinated *Leucadendron* species, while taking into account phylogenetic relatedness, which could provide further insight into the evolutionary modifications that accompany the shift to wind pollination. Finally, I hypothesised that 1) experimental exclusion of insects would have a greater effect on seed production in species with traits associated with insect pollination than in those with traits associated with wind pollination, 2) insects would be more abundant on the inflorescence of *Leucadendron* species with insect pollination traits than on those with wind pollination traits, 3) that shifts from insect to wind pollination would be associated with floral trait modifications including reduction in visual advertising, smaller inflorescences, greater pollen grain production and mobility, reduction in pollen size and sculpturing, reduction in floral scent and a greater degree of sexual dimorphism.

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CHAPTER 2

EXPERIMENTAL EVALUATION OF INSECT VERSUS WIND POLLINATION IN *LEUCADENDRON* (PROTEACEAE)

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EXPERIMENTAL EVALUATION OF INSECT POLLINATION VERSUS WIND POLLINATION IN *LEUCADENDRON* (PROTEACEAE)

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Premise of research. Evolutionary transitions from insect to wind pollination are thought to have occurred many times during the angiosperm radiation. On the basis of floral features, several such transitions have been postulated for *Leucadendron* (Proteaceae), a dioecious genus of 96 taxa confined almost entirely to the Cape Floristic Region. To confirm whether these transitions took place, we carried out experimental tests for wind pollination and sampled insect flower visitors in 15 *Leucadendron* species, representing six clades in the genus.

Methodology. In three clades in which an insect-wind shift has been inferred, we sampled species with traits suggestive of either wind or insect pollination. Plants were bagged with fine-mesh exclusion bags that excluded insects but allowed the passage of airborne pollen. Insects visiting female inflorescences were collected for identification and analysis of their pollen loads.

Pivotal results. We found that insect exclusion had little effect on seed set of five species conforming to the wind pollination syndrome (*L. rubrum*, *L. salicifolium*, *L. dubium*, *L. coniferum*, and *L. teretifolium*), while seed set was strongly reduced by insect exclusion in the remaining 10 species conforming to an insect pollination syndrome. The most common pollinator of the insect-pollinated species was the nitidulid beetle *Pria cinerascens*.

Conclusions. This study provides experimental verification of contrasting insect and wind pollination systems in *Leucadendron* and will thus enable formal phylogenetic tests of adaptive changes in floral traits, such as color and scent, that may be associated with pollination system transitions in this diverse lineage.

Keywords: wind pollination, dioecy, *Leucadendron*, nitidulid, *Pria cinerascens*.

Online enhancement: appendix.

Introduction

Wind pollination has arisen at least 65 times from animal-pollinated ancestors during the evolution of angiosperms (Faegri and van der Pijl 1979; Linder 1998) and is currently found in ~18% of angiosperm families (Ackerman 2000). Wind pollination is thought to evolve when changes to the abiotic or biotic environment result in a decline in pollinator activity or pollinator abundance (Whitehead 1969; Regal 1982; Niklas 1985; Berry and Calvo 1989; Cox 1991; Weller et al. 1998; Goodwillie 1999). Additional ecological circumstances that may favor the evolution of wind pollination include open and dry environments (Whitehead 1969; Niklas 1985; Weller et al. 1998; Ackerman 2000; Culley et al. 2002) and dense populations of congeners (Pannell and Barrett 1998; Davis et al. 2004). Geographically and regionally, wind-pollinated species more commonly occur in higher latitudes and elevations, particularly in temperate forests and grasslands (Regal 1982; Whitehead 1983).

Wind pollination is often associated with a particular suite of morphological traits, including loss of nectaries, small, inconspicuous flowers with fewer (often single) ovules and reduced or absent perianth (Faegri and van der Pijl 1979; Culley et al. 2002; Friedman and Barrett 2008, 2009; Wragg and Johnson 2011). However, there is uncertainty as to whether these traits facilitate the evolution of wind pollination or generally evolve after wind pollination originated in certain lineages (Friedman and Barrett 2008). The pollen grains of wind-pollinated plants often tend to be lighter, drier, smoother, smaller, and more consistently sized than those of insect-pollinated species, presumably because these traits enable effective dispersal by wind (Friedman and Barrett 2008, 2009; Wragg and Johnson 2011).

Wind-pollinated plants often have unisexual flowers; this reduces or (in the case of dioecious species) eliminates self-pollination and thus potentially also detrimental inbreeding depression and pollen discounting (Friedman and Barrett 2008). Overall, phylogenetic analyses suggest that only a few

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trait modifications are necessary for the transition to wind pollination, particularly dry pollen and small flowers (Linder 1998; Wragg and Johnson 2011). Phylogenetically conserved traits, such as dioecy, are more likely to serve as preadaptations (Friedman and Barrett 2008).

Leucadendron R.Br. (Proteaceae) is near-endemic to the Cape Floristic Region and consists of ~96 dioecious taxa (85 species and 11 subspecies; Barker et al. 2004), of which an estimated 89% are insect pollinated. The remaining species are inferred to be wind pollinated (6.6%) or in transition between insect and wind pollination (1.1%) or are too poorly known to make any inferences (3.3%; Williams 1972). A small pollen beetle, *Pria cinerascens* Er. (Nitidulidae), is often associated with *Leucadendron* flowers, particularly the male inflorescences, where they feed on pollen and breed (Williams 1972; Hattingh and Giliomee 1989; Hemborg and Bond 2005). Williams (1972) suggested that at least six species are wind pollinated because of morphological changes such as a lack of conspicuous colored involucral leaves, a reduction of the hypogynous scale (nectaries), increased size of the female stigmatic surface, a lack of distinct floral odor, and showering of pollen when branches are shaken. In a phylogenetic study that used floral syndromes to predict pollination systems, Barker et al. (2004) confirmed Williams' suggestion that insect pollination was ancestral in *Leucadendron*. Linder and Midgley (1996) found that 80.2% of the pollen on the stigmas of *Leucadendron rubrum* Burm. f. was that of *Leucadendron* and probably of this species, highlighting the possible efficiency of wind pollination, although they did not test for the possibility of insect pollination. In a pollination study on several *Leucadendron* species, Hattingh and Giliomee (1989) found that exclusion of insects but not wind had little effect on seed set in *Leucadendron coniferum* (L.) Meisn. To our knowledge, this was the only previous experimental investigation of wind pollination in a *Leucadendron* species. Studies of other genera, such as *Salix* (Vroege and Stelleman 1990; Peeters and Totland 1999), *Schiedea* (Norman et al. 1997), *Croton* (Dominguez et al. 1989), and *Linanthus* (Goodwillie 1999), have shown that plants can have mixed, "ambophilous" pollination systems, in which both wind and insects contribute to seed production, even when floral traits suggest that these species are either insect or wind pollinated. It is therefore necessary to use experimentation to confirm wind pollination, rather than relying on inference from floral syndromes.

The aim of this study was to compare the potential for insect and wind pollination in a sample of *Leucadendron* species

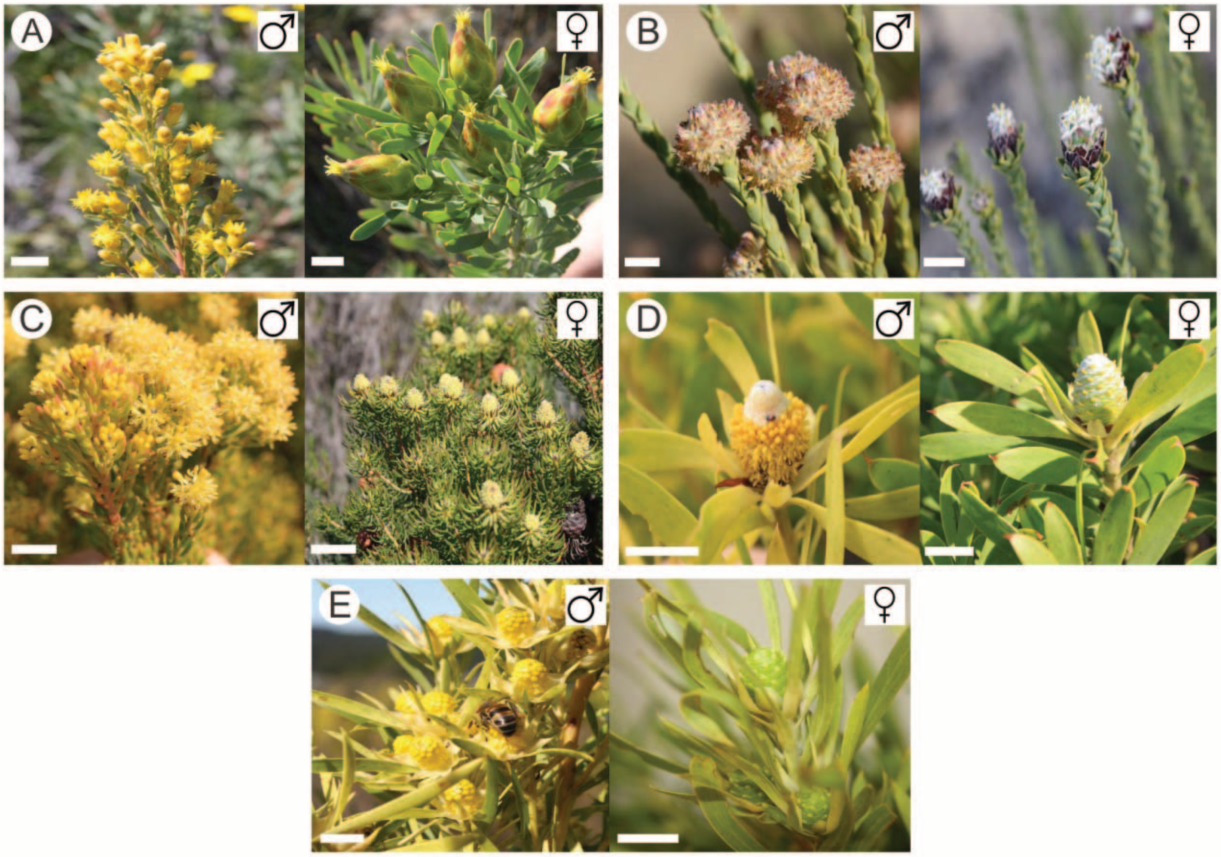

Fig. 1 Male (♂) and female (♀) inflorescences of wind-pollinated *Leucadendron* species: *L. rubrum* (*A*), *L. dubium* (*B*), *L. teretifolium* (*C*), *L. coniferum* (*D*), with a weevil (*Ceutorhynchus* sp. 5) visiting a male inflorescence, and *L. salicifolium* (*E*), with a honeybee (*Apis mellifera*) collecting pollen on the male inflorescence. Scale bars = 10 mm.

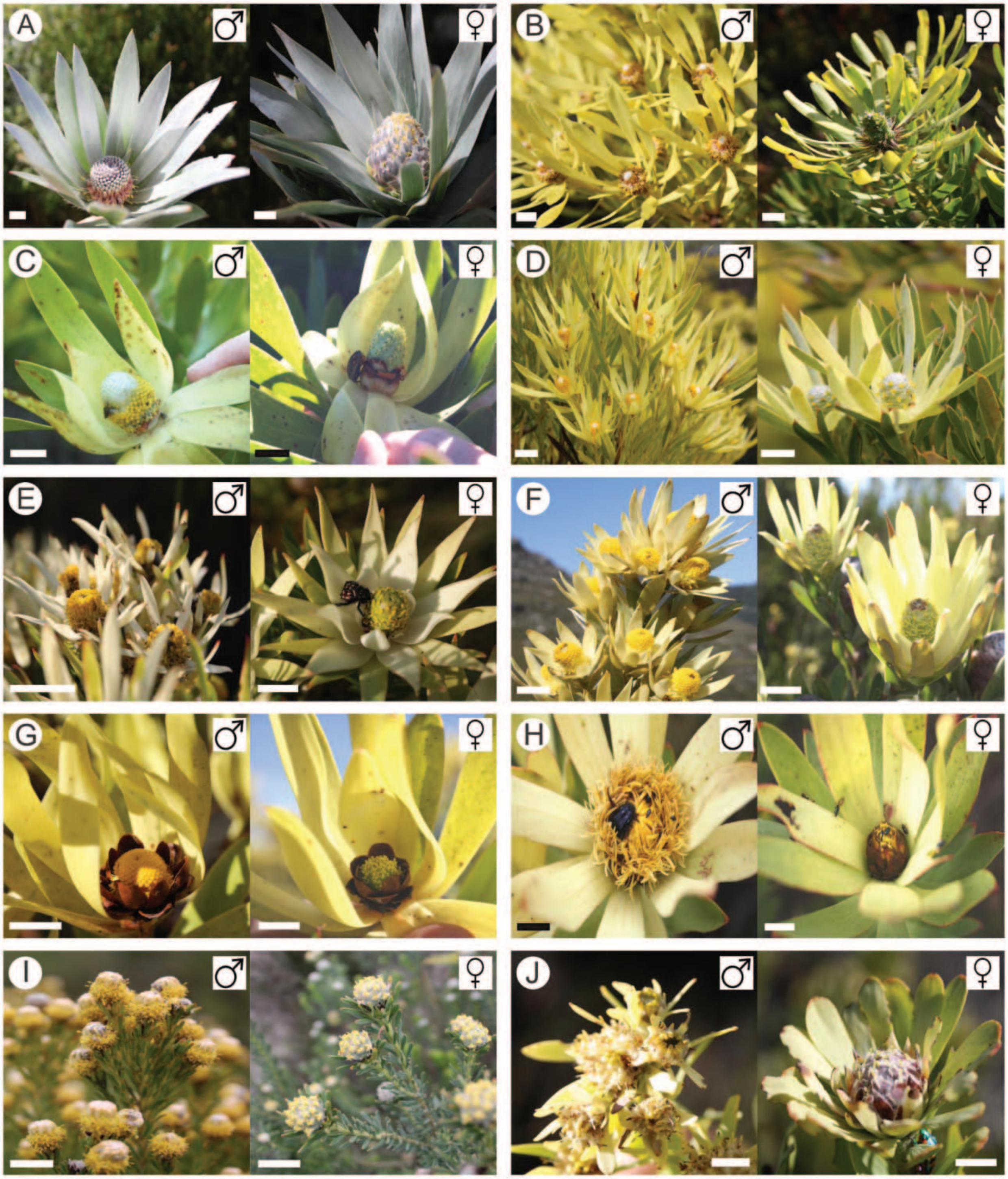

Fig. 2 Male (♂) and female (♀) inflorescences of insect-pollinated *Leucadendron* species: *L. argenteum* (*A*), *L. platyspermum* (*B*), *L. laureolum* (*C*), with a bug (*Solenostethium* sp. 1) visiting a female inflorescence, *L. xanthoconus* (*D*), *L. spissifolium* ssp. *spissifolium* (*E*), with a monkey beetle (*Peritrichia pseudoplebeja*) on the female inflorescence, *L. uliginosum* ssp. *uliginosum* (*F*), *L. microcephalum* (*G*), *L. sessile* (*H*), with monkey beetles (*Anisonyx ursus*) feeding on the pollen in a male inflorescence and several *Chirodica* sp. 1 visiting the female inflorescence, *L. linifolium* (*I*), and *L. pubescens* (*J*). Scale bars = 10 mm.

chosen to be representative of as many clades as possible. We asked whether experimental exclusion of insects would have a greater effect on seed production in species with insect-pollination traits than in those with wind-pollination traits. We also surveyed insect visitors to establish their identity and pollen loads and to compare their abundance between putatively insect- and wind-pollinated species.

Material and Methods

Study Species and Sites

We studied 15 *Leucadendron* species, in total, representing six clades, throughout the southwestern Cape (South Africa) during their flowering periods, from winter to late spring (table A1; tables A1–A4 available online) in 2011 and 2012. In clades for which the insect-wind transition in pollination systems had been inferred (Williams 1972), we selected species with traits suggestive of either wind or insect pollination, as well as three species from clades with no presumed wind-pollinated species, for the purpose of further comparisons (table A1). None of the 15 *Leucadendron* species studied showed female-biased sex ratios (M. R. Welsford, personal observation), thus indicating that apomixis (asexual reproduction) does not occur in these species (apomictic dioecious species produce only female offspring; Bayer and Stebbins 1983). Vouchers of these plant species are deposited in the Bolus Herbarium, University of Cape Town.

Selective-Exclusion Experiment

To evaluate the effect of wind and insects on seed set of the 15 *Leucadendron* species, we applied two treatments to immature virgin female inflorescences: (1) leaving them open to insects and wind pollination and (2) bagging with fine-mesh nylon material that excluded insects but allowed access by wind-borne pollen (Wragg and Johnson 2011). This bagging material has an aperture of 300–500 μm, which is smaller than very small insects, such as the key beetle pollinator *Pria cinarescens* (mean length = 1.8 mm; see "Results"), and much larger than *Leucadendron* pollen (25–50 μm; fig. A1, available online). We applied the treatments of open pollination and insect exclusion to 40 randomly chosen female plants of each species. The only exception was *L. argenteum*, where difficulty in accessing cones on the 6-m-tall plants limited the sample size to 20 plants. The cones were retrieved 2 months after the plants flowered, to ensure that seeds did not drop, in the case of nonserotinous species, and also to prevent loss due to predation, in the case of serotinous species. Nevertheless, a few replicates were lost before collection, but these losses did not exceed 8% of the total sample size.

Bagging would have some effect on the patterns of wind pollination around the inflorescence (Culley et al. 2002). To determine whether the fine-mesh insect-exclusion bags allowed *Leucadendron* pollen through its apertures, the bags were tested in a wind tunnel. Microscope slides were coated with sticky, fuchsin-stained gel to capture pollen and placed side by side (10 cm apart) 25 cm downwind from a male inflorescence in a wind tunnel set at 10 km^{-1} for 10 min. For each 10-min run, the bagged microscope slide and the open microscope slide changed sides. Male inflorescences of two species, *L. laureolum* (insect pollinated) and *L. rubrum* (wind pollinated), were tested in the wind tunnel. Pollen deposition per area was analyzed with generalized estimating equations (GEEs) implemented in SPSS 21 (IBM). Each run (10 min) was treated as the subject in the GEE to account for potential nonindependence in the amount of pollen deposited on the paired open and bagged microscope slides in a particular run. Models incorporated an exchangeable correlation matrix, a negative binomial error distribution, and a log link function. Score statistics were used to assess model significance. In this and all other generalized linear models used in this study, marginal means and standard errors were back-transformed from the scale used in the link function, resulting in asymmetric standard errors.

We determined the proportion of flowers that set seed by

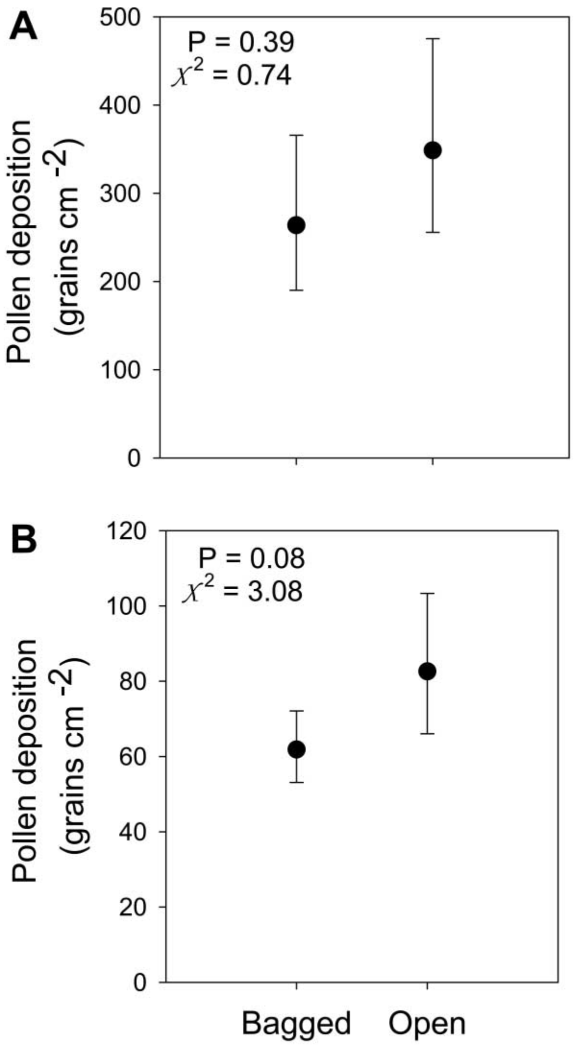

Fig. 3 Mean (±SE) pollen deposition (grains cm^{-2}) on a bagged and an open fuchsin gel–coated microscope slide, placed side by side downwind (25 cm) from a male inflorescence of *Leucadendron rubrum* (*A*; wind pollinated) and *Leucadendron laureolum* (*B*; insect pollinated) in a wind tunnel at 10 km h^{-1}.

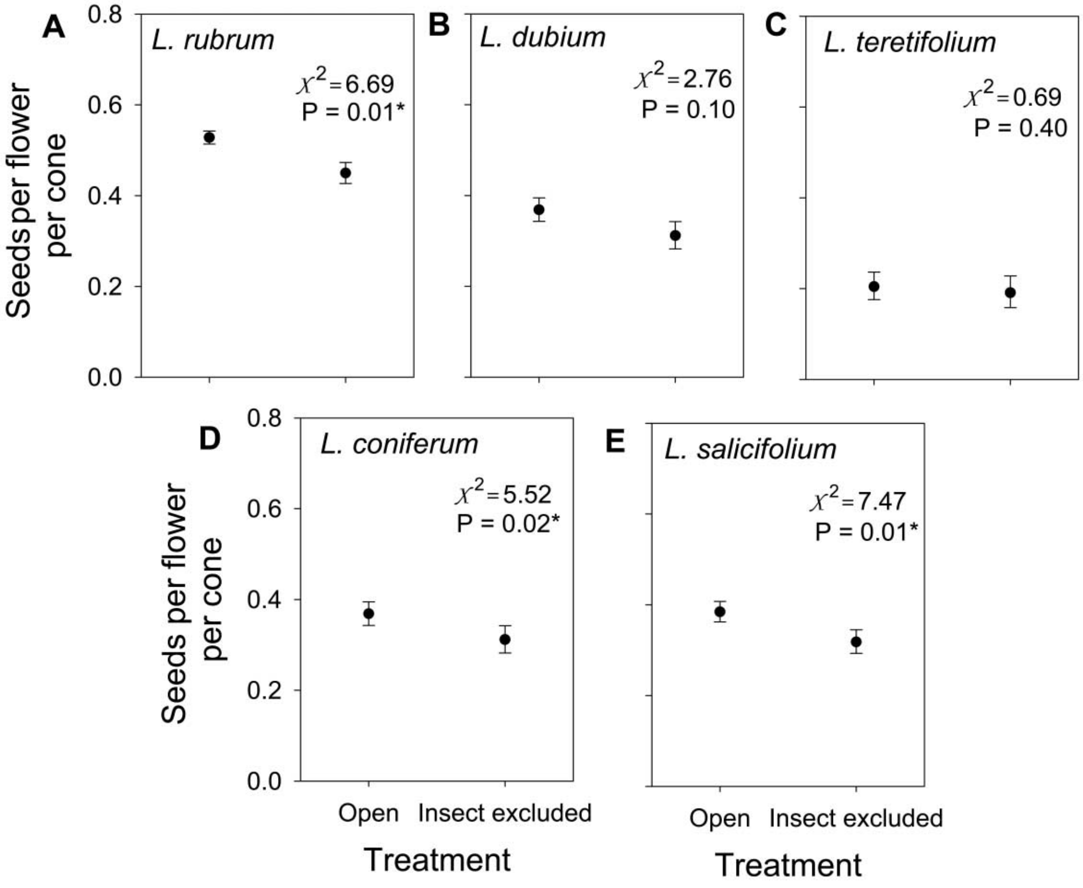

Fig. 4 Mean (±SE) seeds per flower per cone in insect-excluded and open female inflorescences of five wind-pollinated *Leucadendron* species.

counting the number of floral bracts per cone to determine the number of florets per inflorescence and then dissecting the cone to remove the seeds (each flower has a single ovule). In species whose seeds had not fully developed (i.e., did not contain a white, fleshy endosperm), the length of the seeds was used as an indication of viability (the typical size was determined from seeds in the open-pollinated treatment).

The effects of selective exclusion on the mean proportion of flowers that set seed were analyzed with GEE models. Plant identity was treated as the subject to account for nonindependence among treatments that were paired on each plant. Models incorporated an exchangeable correlation matrix, a binomial error distribution (with an events/trials structure), and a logit link function. The dependent variable (events) was the number of seeds per inflorescence as a proportion of the number of ovules (trials). The fixed predictor factor was whether inflorescences were left open or were insect excluded. Score statistics were used to assess model significance.

To compare the overall proportion of flowers that set seed naturally between insect- and wind-pollinated species, we used generalized linear models with a binomial error distribution (events/trials structure) and a logit link function. The dependent variable (events) was the number of seeds per inflorescence as a proportion of the number of ovules (trials). Fixed predictor factors in this model were pollination system and species nested within pollination system.

To determine whether wind pollination and insect pollination in *Leucadendron* are discrete states or occupy various points along a continuum, we determined the frequency distribution of an index of insect pollination for the study species. This index was calculated as 1 − (seed set in the bagged treatment/seed set in the open treatment). Thus, a value of 0 indicates wind pollination (no effect of insect exclusion), and a value of 1 indicates full reliance on insect pollination.

Insect Visitors and Pollinators

To ascertain the abundance, diversity, and pollen loads of insects that visit *Leucadendron* species, floral visitors were sampled for at least one entire day (median = 2) for each species (except *L. argenteum*, which was not sampled) from morning (0800–0900 hours) to late afternoon (1600–1700 hours) on sunny days with little or no wind. For each species, five or six plants of both sexes were examined per hour for floral visitors. The number of inflorescences sampled per hour was consistent for each species and ranged from 10 to 30. Potential insect pollinators were collected from female inflorescences only by shaking the inflorescence into a clear plastic

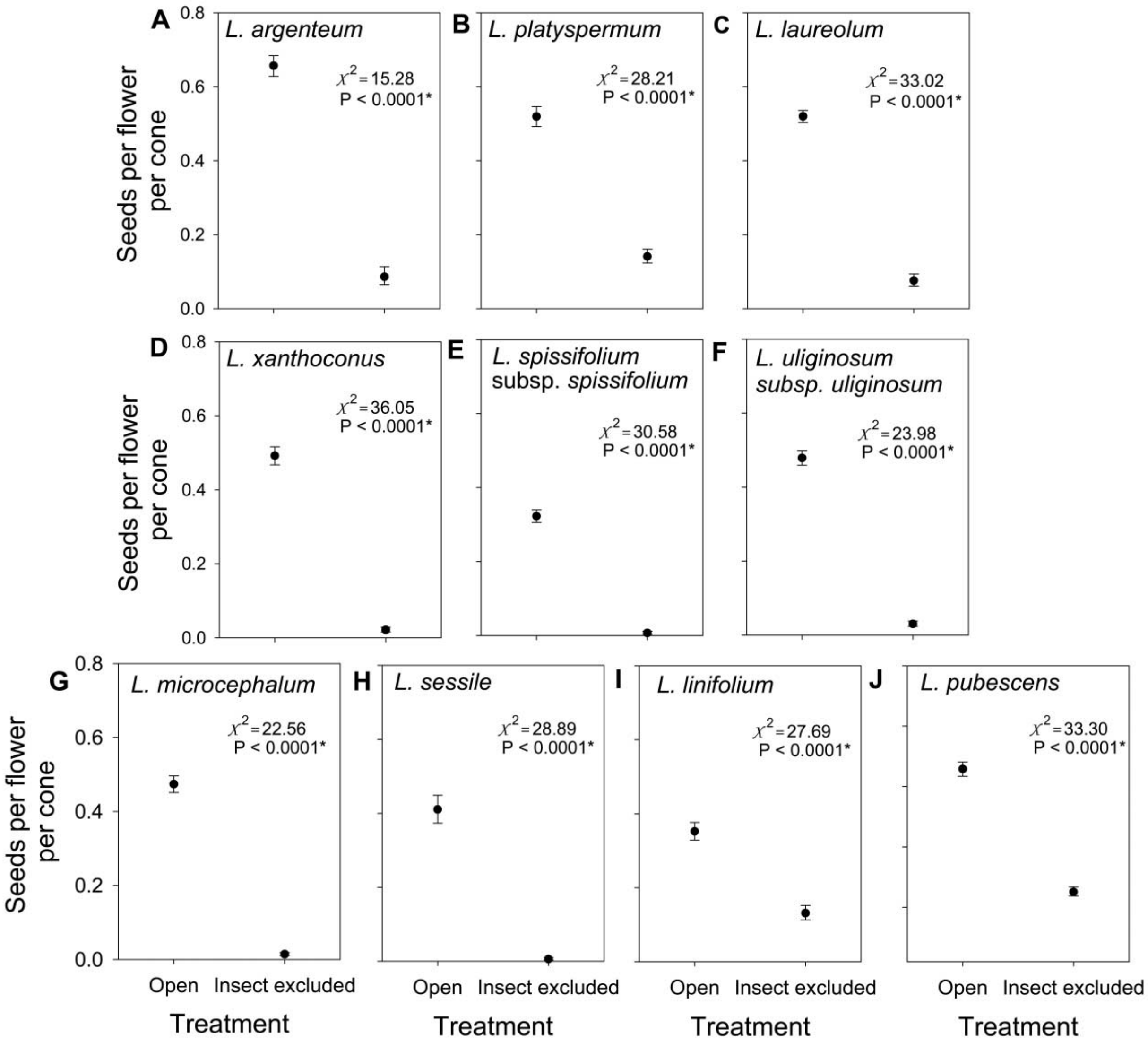

Fig. 5 Mean (±SE) seeds per flower per cone in insect-excluded and open female inflorescences of 10 insect-pollinated *Leucadendron* species.

ziplock bag after first recording whether there was stigmatic contact by the insect(s). Captured insects were placed in vials and then frozen.

Insects collected from female inflorescences were examined for pollen by removing the pollen from their bodies with fuchsin-stained gel to produce permanent slides (Beattie 1971). From a pollen reference slide for each of the *Leucadendron* species, pollen grains were classified as "*Leucadendron*" or "other." Insects were pinned or preserved in 70% ethanol and sent to specialists for identification.

To determine whether the total number of potential insect pollinators and the number of *P. cinerascens* beetles visiting *Leucadendron* inflorescences differed according to pollination system (as determined from the selective-exclusion experiments), sex, or the interaction of pollination system and sex, we used GEE models that incorporated a Poisson error distribution and a log link function. Species identity was treated as the subject to account for potential lack of independence among male and female plants of the same species. Significance was assessed with score statistics. The number of inflorescences of each sex observed per species was used as an offset (a structural predictor with a fixed coefficient of 1) to account for sampling effort and to enable the mean values to be expressed as a rate of insects per inflorescence.

Results

Selective-Exclusion Experiment

The fine-mesh nylon insect-exclusion bags did not significantly reduce pollen deposition on fuchsin gel–coated microscope slides in *L. laureolum* and *L. rubrum* (fig. 3). However, for both species there was a slight decrease in pollen deposition

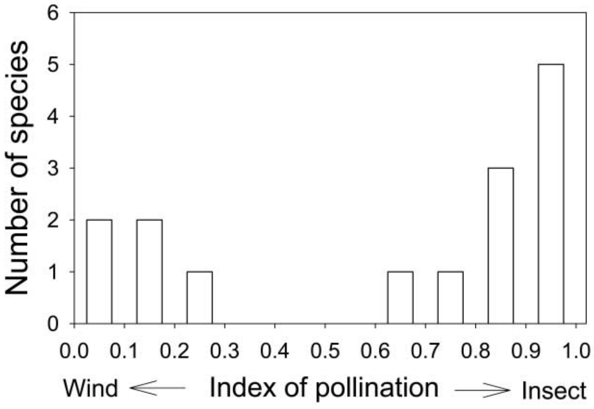

Fig. 6 Frequency distribution of the index of pollination for the number of *Leucadendron* species studied ($n = 15$).

in the bagged microscope slides in comparison to the open microscope slides (fig. 3).

Five *Leucadendron* species with traits suggestive of wind pollination (fig. 1) all showed high levels of seed set within the insect-exclusion bags (fig. 4). In *L. dubium* and *L. teretifolium* there was no significant difference in seed set between the bagged and open treatments, while in *L. coniferum*, *L. rubrum*, and *L. salicifolium* there was a significant reduction in seed set in the bagged treatment, but the differences in means were small (fig. 4).

All 10 *Leucadendron* species with traits suggestive of insect pollination (fig. 2) showed a several-fold and highly significant reduction in seed set in the bagged treatment, compared to the open treatment (fig. 5). Mean natural seed set (i.e., for unbagged female inflorescences) was higher in insect- than in wind-pollinated *Leucadendron* species (0.46 ± 0.04 vs. 0.41 ± 0.06 seeds per flower per cone, $\chi^2 = 62.431$, $P < 0.001$). The frequency distribution for the index of insect pollination was bimodal, indicating that wind and insect pollination are relatively discrete states (fig. 6).

Insect Visitors and Pollinators

Beetles (Coleoptera) were the most frequent visitors to both female and male inflorescences of the 14 *Leucadendron* species examined. On female inflorescences, beetles made up 60% of all floral visitors, followed by Hymenoptera and Hemiptera, totaling 14% each (tables A2, A3).

The most common floral visitor and assumed pollinator of numerous *Leucadendron* species was a small beetle, *Pria cinerascens* (Nitidulidae). Adult *P. cinerascens* are 1.8 ± 0.4 mm ($\bar{x}$ ± SE, $n = 20$) in length and have brown elytra, with a lighter brown head. We observed *P. cinerascens* in abundance on male inflorescences of numerous *Leucadendron* species, where they fed on pollen and copulated (table A4). They also visited female inflorescences and were found to carry large and relatively pure loads of *Leucadendron* pollen and to contact the stigmas (tables 1, 2). They were the main pollinator of *L. laureolum*, *L. xanthoconus*, *L. platyspermum*, *L. microcephalum*, and *L. uliginosum* ssp. *uliginosum* (table 2). *Pria cinerascens* were also observed visiting the presumed wind-pollinated species *L. salicifolium*, *L. coniferum*, and *L. dubium*, not only feeding on pollen of the male inflorescences but also occasionally visiting the female inflorescences (table 2).

Several species of weevils (Coleoptera: Curculionidae), particularly from the genus *Ceutorhynchus*, were observed on the inflorescences of numerous *Leucadendron* species (tables 1, 2), in particular on male inflorescences, where they often reproduced. They carried *Leucadendron* pollen (tables 1, 2), but they did not often contact stigmas and are thus unlikely to be important pollinators.

A monkey beetle species, *Peritrichia pseudoplebeja* (Scarabaeidae: Rutelinae: Hopliini), was the main assumed pollinator of *L. spissifolium* ssp. *spissifolium* (table 1; fig. 2*E*♀). Several monkey beetle species were found visiting *L. sessile* inflorescences, among which the black hairy species *Anisonyx ursus* was most abundant (fig. 2*H*♂). *Anisonyx ursus* was observed predominately visiting male inflorescences during midmorning to late afternoon, where they consumed pollen (fig. 2*H*♂), competed for mates, and copulated. They occasionally visited female inflorescences very briefly (table 1).

The levels of insect visitation were low across all *Leucadendron* species, except for *L. microcephalum* and *L. sessile* (table A4). Although the mean number of potential insect pollinators per inflorescence of both sexes was several-fold higher on insect-pollinated than on wind-pollinated *Leucadendron* species, the variance was considerable, and the means did not differ significantly (fig. 7). The average number of insect pollinators per inflorescence was significantly higher for male inflorescences (fig. 7). Analyses of the number of *P. cinerascens* beetles per inflorescence yielded similar results (pollination system: $\chi^2 = 1.041$, $P = 0.308$; sex: $\chi^2 = 72.855$, $P < 0.001$; interaction of pollination system and sex: $\chi^2 = 0.328$, $P = 0.567$).

Discussion

Our experimental results confirm that both insect and wind pollination systems occur in *Leucadendron* (figs. 4, 5). The five *Leucadendron* species shown here to be wind pollinated (fig. 4) are distributed across three clades in the genus (Barker et al. 2004) and thus probably represent the outcome of independent transitions from insect pollination. An index of insect pollination in *Leucadendron* showed a bimodal distribution (fig. 6), suggesting that wind and insect pollination tend to be discrete states, likely representing stable evolutionary strategies, in the genus.

This relatively discrete distribution of wind and insect pollination systems in *Leucadendron* (fig. 6) probably differs from that in many other genera in which combined wind and insect pollination systems have been postulated (Culley et al. 2002). In *Salix*, for example, some species appear to be completely insect pollinated (Kevan 1972; Sacchi and Price 1988), while others vary in their amount of wind pollination from 2% to 52% (Peeters and Totland 1999). The endemic Hawaiian Islands genus *Schiedea* also shows similar trends, with some species being animal pollinated, some wind pollinated, and some both (Weller et al. 1995, 1998; Norman et al. 1997). In *Leucadendron*, we found that insects make a small contribution to pollination of the otherwise wind-pollinated species

Table 1

Potential Pollinators of Insect-Pollinated *Leucadendron* Species, Indicating Pollen Loads and the Percentage of Individual Insects That Made Stigmatic Contact

Leucadendron species, potential pollinators	No. observed (no. collected)	*Leucadendron* pollen: Grains	*Leucadendron* pollen: %	Stigmatic contact (%)
L. platyspermum:				
Coleoptera:				
Nitidulidae:				
Pria cinerascens	30 (18)	801	54.32 ± 6.57	74
Tenebrionidae:				
Lagriinae sp. 2	4 (1)	282	93.38	25
L. laureolum:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 3	13 (6)	1273	99.77 ± .14	54
Ceutorhynchus sp. 5	3 (3)	272	96.53 ± 1.34	33
Pyctoderes ellipticus	6 (6)	1090	91.58 ± 2.85	83
Nitidulidae:				
P. cinerascens	111 (43)	802	96.74 ± 1.64	71
L. xanthoconus:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 3	4 (4)	256	83.25 ± 7.07	78
Ceutorhynchus sp. 4	4 (4)	239	67.06 ± 6.91	50
Ceutorhynchus sp. 5	5 (5)	284	77.64 ± 6.30	0
Oosomus sp. 1	3 (2)	28	42.53 ± .86	33
Nitidulidae:				
P. cinerascens	18 (5)	217	79.36 ± 1.53	89
L. spissifolium ssp. *spissifolium*:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 6	1 (1)	52	26.13	0
Dermestidae:				
Anthrenus sp. 1	8 (6)	164	52.36 ± 8.82	75
Melyridae:				
Malachiinae sp. 1	5 (4)	282	63.73 ± 8.04	80
Scarabaeidae:				
Anisonyx sp. 1	6 (4)	1880	76.85 ± 11.95	83
Peritrichia pseudoplebeja	16 (10)	4209	79.07 ± 3.33	81
Trichostetha capensis	3 (2)	3252	84.68 ± 1.31	67
L. uliginosum ssp. *uliginosum*:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 2	2 (2)	87	94.07 ± .73	50
Nitidulidae:				
P. cinerascens	51 (18)	212	87.05 ± 3.14	53
L. microcephalum:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 3	3 (3)	76	92.14 ± 5.39	100
Nitidulidae:				
P. cinerascens	22 (16)	812	87.19 ± 1.42	91
L. sessile:				
Coleoptera:				
Chrysomelidae:				
Chirodica sp. 1	472 (30)	270	69.08 ± 4.54	25
Curculionidae:				
Ceutorhynchus sp. 1	1 (1)	21	70	0
Ceutorhynchus sp. 3	4 (0)	0	...	25
Nitidulidae:				
P. cinerascens	11 (0)	0	...	25
Scarabaeidae:				
Anisonyx sp. 2	1 (1)	395	65.5	100
Anisonyx ursus	1 (1)	883	89.37	100

Table 1

(*Continued*)

Leucadendron species, potential pollinators	No. observed (no. collected)	*Leucadendron* pollen		Stigmatic contact (%)
		Grains	%	
L. linifolium:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 2	3 (3)	701	62.39 ± 17.15	67
Tenebrionidae:				
Lagriinae sp. 1	1 (1)	1276	83.34	100
L. pubescens:				
Coleoptera:				
Chrysomelidae:				
Chrysomelidae sp. 1	11 (8)	96	68.51 ± 4.21	27
Curculionidae:				
Ceutorhynchus sp. 3	1 (1)	31	73.81	0

and were also recorded as visitors to their flowers (table 2; fig. 7). Williams (1972) suggested that *L. salicifolium* might be in transition from insect to wind pollination, as the species has traits suggestive of wind pollination (e.g., pollen showers when branches are shaken) as well as traits suggestive of insect pollination (e.g., colorful yellow leaves surrounding the male inflorescence; fig. 1*E*♂). Williams (1972) was also undecided whether *L. coniferum* was insect or wind pollinated or both (ambophilous). Inflorescences of both *L. salicifolium* and *L. coniferum* are frequently visited by *Pria cinerascens* beetles (table 2), supporting Williams's ideas, but the results of our selective-exclusion experiments show that neither species is reliant on insect pollination (fig. 4*D*, 4*E*). Furthermore, the index of wind pollination for these species indicates that they do not occupy intermediate positions along a continuum from full insect to full wind pollination.

Despite the almost complete lack of contribution of insects to pollination of five of the *Leucadendron* species in this study, there was no overall significant difference in insect abundance between inflorescences of these species and those for which insect pollination was important (fig. 7). In part this reflects the enormous variance in insect abundance among species (likely due to the wide range of habitats, from disturbed farmlands to pristine reserves, in which they were studied), which

Table 2

Potential Pollinators of Wind-Pollinated *Leucadendron* Species, Indicating Pollen Loads and the Percentage of Individual Insects That Made Stigmatic Contact

Leucadendron species, potential pollinators	No. observed (no. collected)	*Leucadendron* pollen		Stigmatic contact (%)
		Grains	%	
L. rubrum	...	...	...	...
L. dubium:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 5	15 (5)	655	64.16 ± 12.33	7
Ceutorhynchus sp. 6	5 (1)	16	84.21	20
Nitidulidae:				
Pria cinerascens	22 (6)	409	77.90 ± 4.05	14
L. teretifolium:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 2	6 (1)	9	36	67
L. coniferum:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 3	11 (6)	247	64.05 ± 7.55	36
Ceutorhynchus sp. 4	4 (2)	156	73.73 ± 18.51	50
Ceutorhynchus sp. 5	6 (2)	56	79.81 ± 4.81	33
Nitidulidae:				
P. cinerascens	49 (29)	630	62.29 ± 3.36	73
L. salicifolium:				
Coleoptera:				
Nitidulidae:				
P. cinerascens	20 (18)	585	65.77 ± 3.31	75

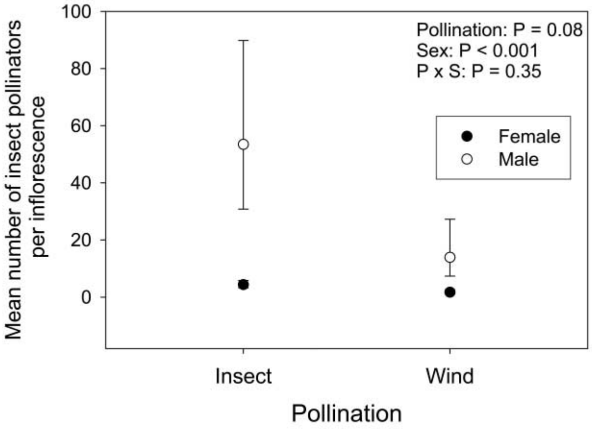

Fig. 7 Mean (±SE) number of insect pollinators per inflorescence per sex in wind- and insect-pollinated *Leucadendron* species. Significance values are given for the main effects and their interaction in a generalized-estimating-equation analysis.

reduced the statistical power to detect differences, but this pattern may also indicate that some of these insects, particularly the nitidulid *P. cinarescens*, gain a benefit from feeding on pollen of wind-pollinated species (table 2). What determines whether a species is insect or wind pollinated may thus be subtle features of floral morphology, particularly stigma and pollen properties, rather than the rate of insect visitation per se. For example, the five wind-pollinated *Leucadendron* species studied have exposed stigmas (fig. 1) and greater pollen mobility compared to species that depend on insects for pollination (M. R. Welsford, unpublished data; fig. 3). In general, these findings indicate that patterns of insect visitation are unreliable as a measure of whether plants are insect or wind pollinated and that selective-exclusion experiments are required to confirm the extent to which insects actually contribute to pollination.

Beetles (Coleoptera) dominated floral visitations and pollination of *Leucadendron* species, with the most common visitor being *P. cinerascens* (Nitidulidae; tables 1, 2). Nitidulids are important pollinators for several plant families, such as Annonaceae (Gottsberger 1999; Silberbauer-Gottsberger et al. 2003; Teichert et al. 2011; Saunders 2012) and Arecaceae (Listabarth 1996; Fava et al. 2011). Our study of a broad range of species supports the findings of Hattingh and Giliomee (1989), who found *P. cinerascens* to be the main pollinator of *L. microcephalum*, *L. salignum*, and *L. laureolum*. Individuals of *P. cinerascens* were found in abundance on male *Leucadendron* inflorescences, where they fed on pollen and reproduced. Importantly for pollination, pollen-carrying individuals also visit female inflorescences (tables 1, 2). However, female inflorescences appear to offer no floral rewards to *P. cinerascens*. Hemborg and Bond (2005) thought that *P. cinerascens* were attracted to female *L. xanthoconus* inflorescences because they offered shelter, an essential resource not offered by male inflorescences. Our observations support this, in that the beetles hide between the inflorescence and the surrounding leaves. However, it is also possible that visits to female inflorescences are mistakes based on scent cues shared between male and female inflorescences. This would parallel the situation in many cycads, where beetles visit male cones for rewards of pollen or cone tissue and essentially visit female cones by mistake because of close matching of the scent of male and female cones (Suinyuy et al. 2013). Proches and Johnson (2009) found that nitidulid beetles that pollinate the female cones of the South African cycad *Stangeria eriopus* (Stangeriaceae) might not be able to discriminate between the sexes, as the scent of their cones is very closely matched.

Our results suggest that two *Leucadendron* species, *L. spissifolium* ssp. *spissifolium* and *L. sessile*, are pollinated primarily by monkey beetles (Hopliini; table 1). *Peritrichia* and *Anisonyx*, the two Hopliini genera to which most of the observed visitor species belong, were placed by Picker and Midgley (1996) in the same guild on the basis of their color preferences and feeding behavior. They suggested that the high frequency of floral visitations and their dense hairs made them important pollinators. Indeed, monkey beetles have been proven to be important pollinators for numerous species in southern Africa (Goldblatt et al. 1998; Steiner 1998; Johnson and Midgley 2001; Van Kleunen et al. 2007).

The relatively high seed set among both insect- and wind-pollinated *Leucadendron* species (±40% each) suggests that both wind and insect pollination are stable evolutionary strategies in this genus. Similar patterns for high seed set in *Leucadendron* were reported by Collins and Rebelo (1987) and Hattingh and Giliomee (1989). These findings support Midgley and Bond's (1991) suggestion that wind pollination can be highly effective and a viable alternative to insect pollination in angiosperms. These findings with respect to seed set, together with phylogenetic evidence for multiple shifts from insect to wind pollination in *Leucadendron* (Barker et al. 2004), challenge the notion that wind pollination is generally inferior to insect pollination on account of being less targeted in its dispersal of pollen (see Whitehead 1983).

The experimental verification of both insect and wind pollination systems in a broad range of *Leucadendron* species, as reported in this study, creates a platform for future studies of evolutionary transitions in *Leucadendron*. We are currently quantifying spectral reflectance and volatile-emission patterns of *Leucadendron* species in order to conduct a phylogenetic analysis of the floral trait modifications associated with pollination system transitions in *Leucadendron*.

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CHAPTER 2

SUPPLEMENTARY MATERIAL

Appendix from Welsford et al., "Experimental Evaluation of Insect versus Wind Pollination in *Leucadendron* (Proteaceae)"

(Int. J. Plant Sci., vol. 175, no. 3, p. 000)

Supplementary Material

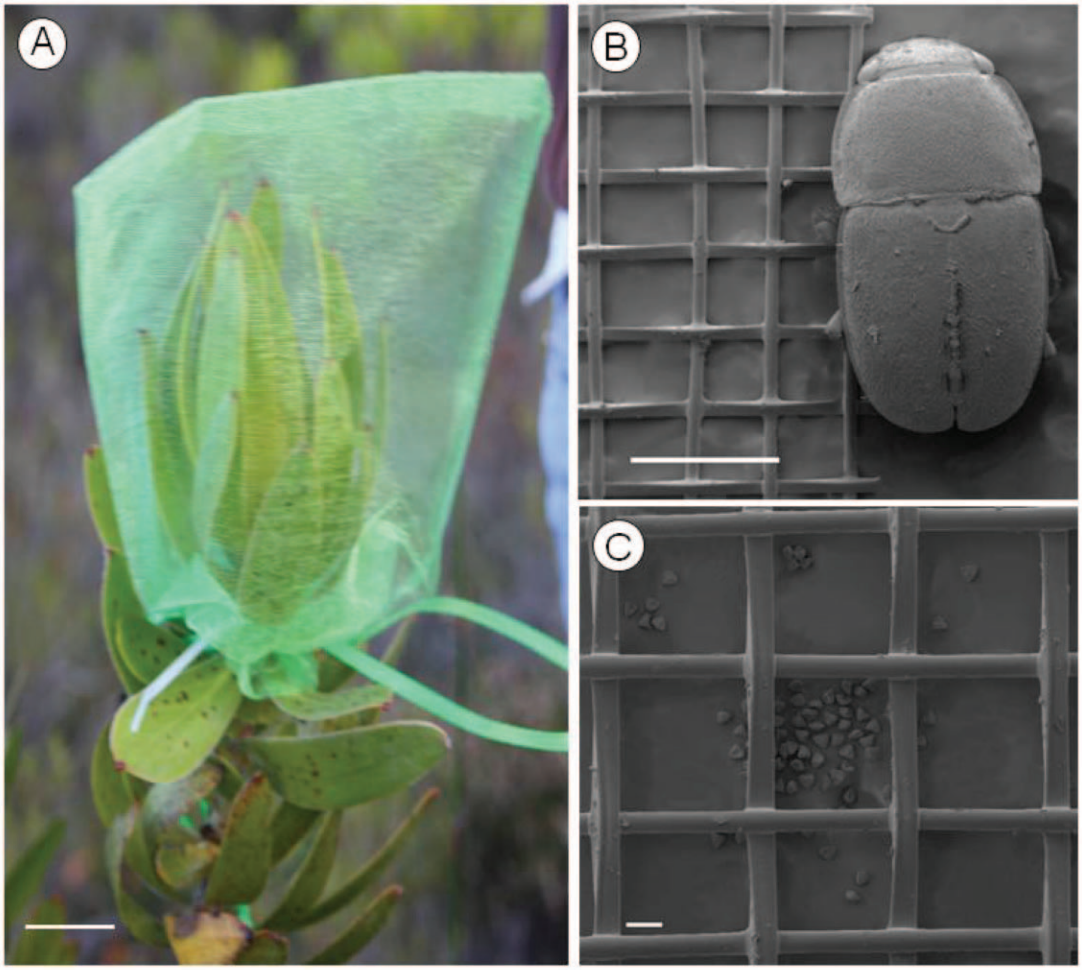

Fig. A1 Fine nylon mesh exclusion bags (unbranded "organza bags" with a drawstring, purchased from Bargain Basket, Pietermaritzburg) that excluded insects but allowed access by wind-borne pollen. *A*, Whole bag on a female *Leucadendron laureolum* inflorescence. *B*, *C*, Scanning electron micrograph of the bagging material in relation to the key beetle pollinator *Pria cinerascens* (*B*) and with scatterings of *Leucadendron rubrum* pollen (*C*). Scale bars = 20 mm (*A*), 1 mm (*B*), and 100 μm (*C*).

Table A1. Site and Coordinate Details of the 15 *Leucadendron* Species Sampled in This Study, Grouped by Clade

Leucadendron species	Clade	Subsection[a]	Study sites	Coordinates
L. argenteum (L.) R.Br.	II	*Leuc.*	Table Mountain Nature Reserve	33°57′S, 18°27′E
L. rubrum Burm. f.[b]	II	*Leuc.*	Table Mountain Nature Reserve	33°57′S, 18°23′E
L. dubium (H. Buek ex Meisn.) E. Phillips & Hutch[b]	II	*Vill.*	Cederberg Wilderness Area	32°24′S, 19°11′E
L. platyspermum R.Br.	III	*Comp.*	Stanford farmlands	34°27′S, 19°33′E
L. teretifolium (Andrews) I. Williams[b]	III	*Comp.*	Caledon farmlands	34°18′S, 19°20′E
L. laureolum (Lam.) Fourc.	V	*Alat.*	Silvermine Nature Reserve	34°05′S, 18°24′E
L. xanthoconus (Kuntze) K.Schum	V	Alat.	Silvermine Nature Reserve	34°05′S, 18°24′E
L. spissifolium (Salisb. ex Knight) I. Williams ssp. *spissifolium*	V	*Alat.*	Limietberg Nature Reserve	33°37′S, 19°06′E
L. coniferum (L.) Meisn[b]	V	*Alat.*	Cape Point Nature Reserve	34°18′S, 18°26′E
L. uliginosum R.Br. ssp. *uliginosum*	V	*Trig.*	Witfontein Nature Reserve	33°52′S, 22°24′E
L. salicifolium (Salisb.) I. Williams[b]	V	*Trig.*	Hottentots Holland Nature Reserve	34°08′S, 18°56′E
L. microcephalum (Gand.) Gand. & Schinz	V	*Brun.*	Hottentots Holland Nature Reserve	34°05′S, 19°03′E
L. sessile R.Br.	VI	*Nuc.*	Elandskloof Nature Reserve	34°08′S, 18°55′E
L. linifolium (Jacq.) R.Br.	VII	*Vill.*	Stanford farmlands	34°25′S, 19°30′E
L. pubescens R.Br.	VIII	*Memb.*	Sawadee-Cederberg farm	32°20′S, 18°59′E

Note. Clades per Barker et al. (2004).

[a] *Alat.* = *Alata*, *Brun.* = *Brunneobracteata*, *Comp.* = *Compressa*, *Leuc.* = *Leucadendron*, *Memb.* = *Membranacea*, *Nuc.* = *Nucifera*, *Vill.* = *Villosa*.

[b] The five *Leucadendron* species with traits suggestive of wind pollination.

Table A2. Insect Visitors on Female Inflorescences of Insect-pollinated *Leucadendron* Species, Indicating Pollen Loads and the Percentage of Individuals That Made Stigmatic Contact

Leucadendron species, floral visitors	No. observed (No. collected)	*Leucadendron* pollen: Grains	*Leucadendron* pollen: %	Stigmatic contact (%)
L. platyspermum:				
Coleoptera:				
Anthicidae:				
Notoxus sp. 1	1 (1)	4	50	100
Curculionidae:				
Ceutorhynchus sp. 2	2 (0)	0	...	50
Nitidulidae:				
Pria cinerascens	30 (18)	801	54.32 ± 6.57	74
Tenebrionidae:				
Lagriinae sp. 2	4 (1)	282	93.38	25
L. laureolum:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 3	13 (6)	1273	99.77 ± 0.14	54
Ceutorhynchus sp. 5	3 (3)	272	96.53 ± 1.34	33
Pyctoderes ellipticus	6 (6)	1090	91.58 ± 2.85	83
Nitidulidae:				
P. cinerascens	111 (43)	802	96.74 ± 1.64	71
Hemiptera:				
Anthocoridae:				
Xylocoris sp. 1	189 (61)	414	87.49 ± 2.40	14
Pentomatidae:				
Antestiopsis orbitalis	2 (2)	92	77.56 ± 1.17	100
Scutelleridae:				
Solenostethium sp. 1	10 (4)	99	81.59 ± 5.35	80
Thysanoptera:				
Phlaeothripidae:				
Phlaeothripidae sp. 1	8 (0)	0	...	12
L. xanthoconus:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 3	4 (4)	256	83.25 ± 7.07	78
Ceutorhynchus sp. 4	4 (4)	239	67.06 ± 6.91	50
Ceutorhynchus sp. 5	5 (5)	284	77.64 ± 6.30	0
Oosomus sp. 1	3 (2)	28	42.53 ± 0.86	33

Table A2 (*Continued*)

Leucadendron species, floral visitors	No. observed (No. collected)	*Leucadendron* pollen		Stigmatic contact (%)
		Grains	%	
Nitidulidae:				
P. cinerascens	18 (5)	217	79.36 ± 1.53	89
Hemiptera:				
Anthocoridae:				
Xylocoris sp. 1	32 (19)	103	53.58 ± 4.18	50
Isometopidae:				
Isometopidae sp. 1	1 (1)	5	55.56	0
Diptera:				
Sciaridae:				
Bradysia sp. 1	1 (1)	84	96.55	100
L. spissifolium ssp. *spissifolium*:				
Coleoptera:				
Chrysomelidae:				
Alticinae sp. 1	2 (1)	19	36.54	50
Alticinae sp. 2	2 (1)	20	54.05	100
Eumolpinae sp. 1	3 (3)	119	53.54 ± 9.36	100
Curculionidae:				
Ceutorhynchus sp. 6	1 (1)	52	26.13	0
Gymnetron sp. 1	1 (1)	79	70.54	100
Dermestidae:				
Anthrenus sp. 1	8 (6)	164	52.36 ± 8.82	75
Melyridae:				
Malachiinae sp. 1	5 (4)	282	63.73 ± 8.04	80
Scarabaeidae:				
Anisonyx sp. 1	6 (4)	1880	76.85 ± 11.95	83
Peritrichia pseudoplebeja	16 (10)	4209	79.07 ± 3.33	81
Trichostetha capensis	3 (2)	3252	84.68 ± 1.31	67
Hemiptera:				
Anthocoridae:				
Xylocoris sp. 1	7 (5)	82	58.74 ± 3.48	0
Hymenoptera:				
Formicidae:				
Formicidae sp. 1	1 (1)	101	77.69	0
Diptera:				
Empididae:				
Platypalpus sp. 1	3 (1)	0	...	33
Muscidae:				
Helina sp. 1	1 (1)	5	71.43	100
L. uliginosum ssp. *uliginosum*:				
Coleoptera:				
Chrysomelidae:				
Chirodica sp. 1	1 (0)	0	...	0
Coccinellidae:				
Coccinellidae sp. 2	2 (0)	0	...	100
Curculionidae:				
Ceutorhynchus sp. 2	2 (2)	87	94.07 ± 0.73	50
Nitidulidae:				
P. cinerascens	51 (18)	212	87.05 ± 3.14	53
Diptera:				
Calliphoridae:				
Calliphoridae sp. 2	4 (2)	98	72.19 ± 0.22	100
Thysanoptera:				
Phlaeothripidae:				
Phlaeothripidae sp. 1	10 (0)	0	...	20
L. microcephalum:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 3	3 (3)	76	92.14 ± 5.39	100
Nitidulidae:				
P. cinerascens	22 (16)	812	87.19 ± 1.42	91
Thysanoptera:				
Phlaeothripidae:				

Table A2 (*Continued*)

Leucadendron species, floral visitors	No. observed (No. collected)	*Leucadendron* pollen		Stigmatic contact (%)
		Grains	%	
Phlaeothripidae sp. 1	4 (0)	0	...	25
L. sessile:				
Coleoptera:				
Buprestidae:				
Buprestidae sp. 1	1 (1)	27	81.81	0
Chrysomelidae:				
Alticinae sp. 1	2 (2)	101	93.77 ± 1.97	100
Chirodica sp. 1	472 (30)	270	69.08 ± 4.54	25
Curculionidae:				
Ceutorhynchus sp. 1	1 (1)	21	70	0
Ceutorhynchus sp. 3	4 (0)	0	...	25
Nitidulidae:				
P. cinerascens	11 (0)	0	...	25
Scarabaeidae:				
Anisonyx sp. 2	1 (1)	395	65.5	100
Anisonyx ursus	1 (1)	883	89.37	100
Diptera:				
Tephtitidae:				
Spathulina sp. 1	2 (1)	9	81.82	0
L. linifolium:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 2	3 (3)	701	62.39 ± 17.15	67
Tenebrionidae:				
Lagriinae sp. 1	1 (1)	1276	83.34	100
Diptera:				
Calliphoridae:				
Calliphoridae sp. 1	1 (1)	18	54.55	100
L. pubescens:				
Coleoptera:				
Chrysomelidae:				
Chrysomelidae sp. 1	11 (8)	96	68.51 ± 4.21	27
Curculionidae:				
Ceutorhynchus sp. 3	1 (1)	31	73.81	0
Oosomus sp. 1	2 (2)	55	80.16 ± 8.73	50
Melyridae:				
Malachiinae sp. 1	3 (1)	28	93.33	33
Melyrus sp. 1	2 (1)	128	86.49	100
Nitidulidae:				
Carpophilus sp. 1	1 (1)	7	70	100
Diptera:				
Tephritidae:				
Desmella sp. 1	4 (1)	18	72.00	50
Hemiptera:				
Scutelleridae:				
Calidea dregii	1 (1)	8	12.12	0

Note. *Leucadendron argenteum* was not observed for insect visitors because of its height (>6 m).

Table A3. Insect Visitors on Female Inflorescences of Wind-Pollinated *Leucadendron* Species, Indicating Pollen Loads and the Percentage of Individuals That Made Stigmatic Contact

Leucadendron species, floral visitors	No. observed (No. collected)	*Leucadendron* pollen		Stigmatic contact (%)
		Grains	%	
L. rubrum	...	...	...	...
L. dubium:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 2	1 (1)	3	60	100
Ceutorhynchus sp. 5	15 (5)	655	64.16 ± 12.33	7
Ceutorhynchus sp. 6	5 (1)	16	84.21	20

Table A3 (*Continued*)

Leucadendron species, floral visitors	No. observed (No. collected)	*Leucadendron* pollen		Stigmatic contact (%)
		Grains	%	
Sibinia sp. 1	1 (1)	46	75.41	100
Nitidulidae:				
Pria cinerascens	22 (6)	409	77.90 ± 4.05	14
Diptera:				
Bibionidae:				
Dilophus sp. 1	6 (1)	104	91.23	67
Hymenoptera:				
Formicidae:				
Formicidae sp. 2	2 (2)	38	77.52 ± .25	0
Thysanoptera:				
Phlaeothripidae:				
Phlaeothripidae sp. 1	108 (31)	306	62.95 ± 3.29	23
Thripidae:				
Thripidae sp. 1	47 (13)	112	61.14 ± 2.78	17
L. teretifolium:				
Coleoptera:				
Curculionidae:				
Ceutorhynchus sp. 2	6 (1)	9	36	67
Melyridae:				
Dasytinae sp. 1	1 (1)	44	86.27	100
Diptera:				
Scathophagidae:				
Scathophaga sp. 1	20 (8)	1033	68.89 ± 9.40	100
L. coniferum:				
Coleoptera:				
Apionidae:				
Apiomorphus eximius	1 (1)	91	76.47	0
Coccinellidae:				
Coccinellidae sp. 1	1 (0)	0	...	100
Curculionidae:				
Ceutorhynchus sp. 3	11 (6)	247	64.05 ± 7.55	36
Ceutorhynchus sp. 4	4 (2)	156	73.73 ± 18.51	50
Ceutorhynchus sp. 5	6 (2)	56	79.81 ± 4.81	33
Melyridae:				
Malachiinae sp. 2	4 (2)	15	48.68 ± 1.32	75
Nitidulidae:				
P. cinerascens	49 (29)	630	62.29 ± 3.36	73
Diptera:				
Empididea:				
Empididae sp. 1	2 (2)	8	80 ± 20	100
Sciaridae:				
Bradysia sp. 1	1(1)	3	75	0
Sciaridae sp. 1	1(1)	0	...	100
Hemiptera:				
Anthocoridae:				
Xylocoris sp. 1	3 (1)	29	64.44	67
Thysanoptera:				
Phlaeothripidae:				
Phlaeothripidae sp. 1	2 (2)	14	53.57 ± 3.57	100
L. salicifolium:				
Coleoptera:				
Coccinellidae:				
Coccinellidae sp. 3	2 (2)	145	85.56 ± 2.37	50
Nitidulidae:				
P. cinerascens	20 (18)	585	65.77 ± 3.31	75

Table A4. Mean Number of All Insects and *Pria cinerascens* Individuals Observed per Inflorescence of Both Sexes for Each *Leucadendron* Species

Pollination system, *Leucadendron* species, sex	No. inflorescences observed	Average no. insects per inflorescence (±SE)	No. of *P. cinerascens*
Insect:			
L. platyspermum:			
Male	540	.30 ± .03	153
Female	270	.16 ± .03	30
L. laureolum:			
Male	650	.71 ± .06	314
Female	650	.60 ± .05	111
L. xanthoconus:			
Male	1680	1.69 ± .05	414
Female	840	1.11 ± .03	18
L. spissifolium ssp. *spissifolium*:			
Male	480	.09 ± .02	1
Female	240	.25 ± .03	0
*L. uliginosum*ssp. *uliginosum*:			
Male	960	.89 ± .04	840
Female	480	1.15 ± .05	51
L. microcephalum:			
Male	420	4.75 ± .22	1705
Female	210	.12 ± .03	22
L. sessile:			
Male	631	7.58 ± .41	1166
Female	624	.79 ± .08	11
L. linifolium:			
Male	960	.01 ± .003	1
Female	960	.01 ± .003	0
L. pubescens:			
Male	1350	.09 ± .01	0
Female	900	.04 ± .01	0
Wind:			
L. rubrum:			
Male	720	.006 ± .003	1
Female	480	.004 ± .003	0
L. dubium:			
Male	1080	.35 ± .02	68
Female	1080	.20 ± .02	22
L. teretifolium:			
Male	960	.02 ± .01	1
Female	960	.05 ± .004	0
L. coniferum:			
Male	960	1.16 ± .05	971
Female	480	.20 ± .03	49
L. salicifolium:			
Male	960	.13 ± .01	115
Female	960	.02 ± .01	20

CHAPTER 3

FLORAL TRAIT EVOLUTION ASSOCIATED WITH SHIFTS FROM INSECT TO WIND POLLINATION IN DIOECIOUS *LEUCADENDRON* (PROTEACEAE)

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Transitions between pollination systems have made a major contribution to angiosperm floral diversification. Transitions from animal- to wind pollination have occurred in many lineages and have been linked to various floral modifications, but these have seldom been assessed in a phylogenetic framework. We compared floral traits of twelve insect-pollinated and five wind-pollinated species of the dioecious genus *Leucadendron* (Proteaceae) to determine whether transitions from animal to wind-pollination were accompanied by modifications of pollination-relevant floral traits, such as, floral morphology, visual and olfactory cues and degree of dimorphism between sexes. In a wind tunnel, pollen grains of wind-pollinated species were found to be more motile than those of insect-pollinated species. Phylogenetic analyses suggest that transitions from insect to wind pollination occurred at least four times during the diversification of *Leucadendron* and, in comparison to insect-pollinated congeners, wind-pollinated *Leucadendron* species are characterized by increased production of smaller pollen grains, greater inflorescence density, reduction in spectral contrast between subtending and background leaves, reduced volatile emissions and, a greater degree of sexual dimorphism in visual and olfactory cues. These results offer key insights into the modification of floral traits involved in the transition from insect to wind pollination.

KEY WORDS: Ancestral state reconstruction, pGEE, phylogenetic signal, gas chromatograph-mass spectrometry, sexual dimorphism, wind tunnel, spectral reflectance.

Shifts between pollination systems are often associated with speciation, as these transitions usually have implications for both reproductive isolation and divergence of the floral phenotype (Grant and Grant 1965; Stebbins 1970; Johnson 2006; Campbell 2008). Such shifts are considered to have made a major contribution to angiosperm diversification, as evidenced by studies that examined the frequency of historical pollination-system shifts in a phylogenetic context (van der Niet and Johnson 2012). Pollination-system shifts are likely precipitated by spatio-temporal variation in the abundance of pollinators (Grant and Grant 1965; Stebbins 1970). Besides the well-documented evolution of self-fertilization (Lloyd 1979; Schoen et al. 1996; Kalisz et al. 1999), a possible outcome of pollinator limitation of fitness in animal-pollinated plants is a shift to wind pollination (Cox 1991; Weller et al. 1998; Goodwillie 1999). This shift may be especially likely when the plants occur in open habitats with low humidity and/or low precipitation, and have floral morphology that “pre-adapts” them to such shifts (see Culley et al. 2002). Evolutionary transitions from animal to wind pollination have been recorded in ~18% of angiosperm families (Vroege and Stelleman 1990; Weller et al. 1998; Blattner and Kadereit 1999; Ackerman 2000; Manos et al. 2001; Culley et al. 2002; Welsford et al. 2014). While wind pollination was once thought to be relatively ineffective compared to animal pollination, on account of less targeted and more wasteful pollen dispersal (Whitehead 1983; Cox 1991), the plethora of shifts from animal to wind pollination suggests that wind pollination must enhance fitness under certain conditions (Midgley and Bond 1991; Welsford et al. 2014).

Transitions from animal to wind pollination are associated with modifications to suites of floral traits, as evidenced by broad patterns of convergent evolution (Whitehead 1969; Faegri and van der Pijl 1979; Linder 1998; Reeves and Olmstead 1998; Goodwillie 1999; Linder 2000). Animal-pollinated flowers are typically large, fragrant, colourful, and produce a food reward for pollinators. By comparison, wind-pollinated flowers are typically small (with highly reduced perianth), unscented, inconspicuous, and do not produce food rewards (Faegri and van der Pijl 1979; Linder 1998; Culley et al. 2002; Friedman and Barrett 2008, 2009; Wragg and Johnson 2011). Many of the floral trait modifications of wind-pollinated species can be linked to the requirements for dispersal and capture of wind-borne pollen (Whitehead 1983; Culley et al. 2002), such as dry, small, smooth, light, and consistently sized pollen grains and large stigmas (Linder 1998; Wragg and Johnson 2011). Phylogenetic analyses suggest that the key trait changes associated with the transition from animal to wind pollination are a reduction of flower size and a transition to dry pollen (Linder 1998; Wragg and Johnson 2011). Furthermore, a disproportionate number of wind-pollinated plants are dioecious (Renner and Ricklefs 1995). This sex distribution promotes outcrossing and may serve as a pre-adaptation for wind pollination in certain lineages (Culley et al 2002; Friedman and Barrett 2008).

Leucadendron R.Br. (Proteaceae) is a dioecious woody genus largely endemic to the Cape Fynbos and comprises 96 taxa, (85 species and 11 subspecies; Barker et al. 2004), of which ~ 89% are suggested to be insect-pollinated and ~ 6.6% are inferred to be wind-pollinated based on floral traits (Williams 1972). These wind-pollinated species tend to have reduced or absent hypogynous scales (nectaries), no discernible floral scent to humans, inconspicuously coloured subtending leaves surrounding the inflorescence, and pollen that is released into the air upon shaking of branches (Williams 1972). Welsford et al. (2014) recently established the pollination systems of 15 *Leucadendron* species (five wind-pollinated and ten insect-pollinated) using selective exclusion experiments. The main pollinator of insect-pollinated species is a small nitidulid beetle, *Pria cinerascens* (Hattingh and Giliomee 1989; Hemborg and Bond 2005; Welsford et al. 2014), which feeds mainly on pollen from male inflorescences. In an earlier phylogenetic reconstruction of shifts in *Leucadendron* using floral syndromes (rather than confirmed pollination systems), Barker et al. (2004) concluded that insect pollination was always ancestral to wind pollination.

Studies have investigated the degree of sexual dimorphism in species of *Leucadendron*, focusing mainly on leaf size (Bond and Midgley 1988; Midgley 2010). Midgley (2010) suggested that among flowering plants, the genus might have the highest degree of gender-linked leaf dimorphism. Furthermore, putatively wind-pollinated *Leucadendron* species tend to have a greater degree of leaf size dimorphism than the putatively insect-pollinated species (Bond and Midgley 1988), possibly because the structural requirements to disperse and receive wind-borne pollen differ greatly (Lloyd and Webb 1977, 1986; Friedman and Harder 2004). Alternatively, wind-pollinated species may be more sexually dimorphic as they are not constrained by a need for similarity in signals between males and females (in the insect-pollinated species, beetles obtain rewards only from male inflorescences, and pollinate the rewardless female inflorescence when they visit them by mistake). Indeed, in the absence of these constraints, the wind-pollinated species, *Leucadendron rubrum* Burm.f., offers a striking case of sexual dimorphism in inflorescence production and plant architecture (Friedman and Barrett 2009; Barrett and Hough 2013). However, Hemborg and Bond (2005) suggested that sexual dimorphism in

L. xanthoconus (Kuntze) K. Schum., an insect-pollinated species, is a result of its nitidulid beetle pollinator, *P. cinerascens*, obtaining different rewards in male and female inflorescences (pollen from males and shelter in females). Harris and Pannell (2010) found that the degree of sexual dimorphism was more pronounced in serotinous species, in which females are less branched than males to compensate for the costs of producing cones.

Comparison of floral traits of experimentally confirmed insect- and wind-pollinated *Leucadendron* species could offer further insight into the evolutionary trait changes, including degree of sexual dimorphism, that accompany the transition from insect to wind pollination. We therefore investigated floral trait changes associated with the transition from insect to wind pollination in 17 *Leucadendron* species -- five wind-pollinated and twelve insect-pollinated (Hattingh and Giliomee 1989; Welsford et al. 2014) -- while accounting for phylogenetic relatedness among the species. We investigated the following predictions based on evolutionary shifts in floral traits from animal to wind pollination: 1) pollen grains of wind-pollinated species would be more motile in wind than those of insect-pollinated species; 2) pollen grains of wind-pollinated species would be smaller and more numerous than those of insect-pollinated species; 3) insect-pollinated species would have more flowers per inflorescence and larger inflorescences than those of wind-pollinated species to attract pollinators; 4) wind-pollinated species will have greater inflorescence density (inflorescences per branch) for effective pollen dispersal and greater possibility of receiving pollen; 5) the colour contrast between subtending leaves surrounding inflorescences and background green leaves would be greater for insect-pollinated species, given that in insect-pollinated species the subtending leaves are often yellow and “showy”; 6) emission rates of volatiles and the number of compounds emitted from flowers and inflorescences would be greater for insect-pollinated species; and 7) wind-pollinated species would have a greater degree of sexual dimorphism in pollination-relevant floral traits than insect-pollinated species.

Materials and Methods

STUDY TAXA AND PHYLOGENETIC RELATEDNESS

Seventeen *Leucadendron* species, representing six of the nine clades (Barker et al. 2004), were studied throughout the south-western Cape (South Africa) during peak flowering time (June-December) (Table S1). We sampled five wind-pollinated *Leucadendron* species and twelve insect-pollinated *Leucadendron* species whose pollination systems were established in previous studies (Hattingh and Giliomee 1989; Welsford et al. 2014). One wind- and at least one insect-pollinated species were examined from each of the six clades included here (Table S1). We deposited vouchers of these *Leucadendron* species in the Bolus Herbarium (BOL), University of Cape Town, South Africa.

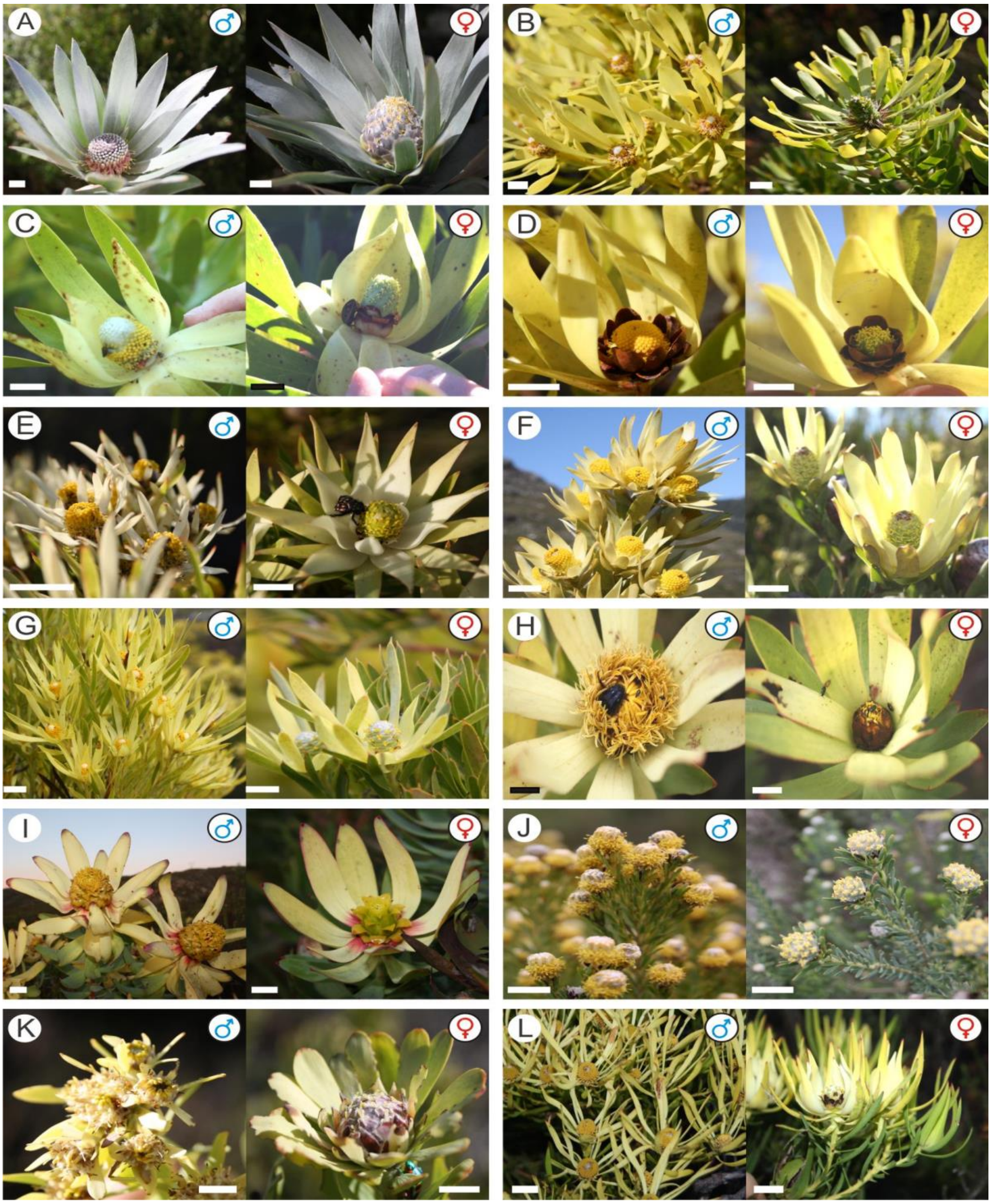

Figure 1. Male (♂) and female (♀) inflorescences of insect-pollinated *Leucadendron* species: (A) *L. argenteum*; (B) *L. platyspermum*; (C) *L. laureolum* (D) *L. microcephalum*;

(E) *L. spissifolium* subsp. *spissifolium*; (F) *L. uliginosum* subsp. *uliginosum*; (G) *L. xanthoconus*; H) *L. sessile*; (H) *L. tinctum*; (I) *L. linifolium*; (K) *L. pubescens*; (L) *L. salignum*. Scale bars: 10 mm.

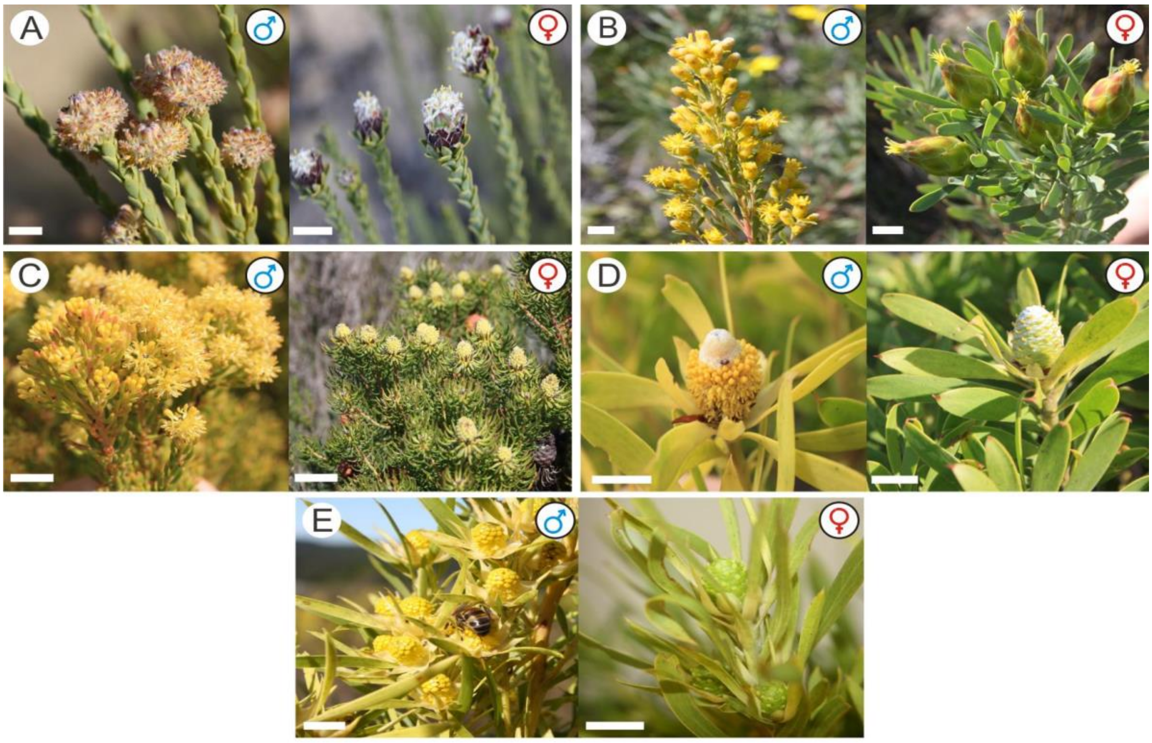

Figure 2. Male (♂) and female (♀) inflorescences of wind-pollinated *Leucadendron* species: (A) *L. dubium*; (B) *L. rubrum*; (C) *L. teretifolium*; (D) *L. coniferum*; (E) *L. salicifolium*. Scale bars: 10 mm.

We inferred phylogenetic relationships from the molecular dataset of *Leucadendron* by Barker et al. (2004), which was reanalysed by Hoffmann (2012) using Bayesian Evolutionary Analysis by Sampling Trees (BEAST, Drummond and Rambaut 2007) to date the phylogeny (Fig. S7). Chronograms included all *Leucadendron* species for which pollination-system data was collected. The recently published phylogeny of *Leucadendron* by Tonnabel et al. (2014) was not used for the analyses in this study as it did not include some of the study species and chronogram topology generally agreed with that of Hoffmann (2012).

POLLEN MOTILITY IN A WIND TUNNEL

We measured the wind-dispersal efficiency of pollen for wind- versus insect-pollinated species in three clades (*L. coniferum* vs. *L. laureolum*, *L. teretifolium* vs. *L. platyspermum* and *L. rubrum* vs. *L. argenteum*) using a tubular wind tunnel (see Fig. S1; cf. Wragg and Johnson 2011). We counted the number of male flowers at anthesis before and again after placing the male inflorescence upwind of three microscope slides coated in sticky fuchsin gel (Beattie 1971), set at three different distances (10, 30 and 90 cm). To reduce turbulence and create laminar flow, air was pulled through the wind tunnel, entering first through thousands of closely spaced horizontal plastic straws. Following the protocol by Wragg and Johnson (2011), we used a randomized block design, where each of four blocks included one run of each species of three wind speeds: 1.39 m s^{-1} (5 km h^{-1}), 2.78 m s^{-1} (10 km h^{-1}), and 5.56 m s^{-1} (20 km h^{-1}). During each run, we exposed a different inflorescence for 10 minutes in the wind tunnel, and counted the number of pollen grains captured per microscope slide.

We implemented a generalized estimating equation (GEE) in SPSS 21 (IBM Corp.) that incorporated an autoregressive (AR1) correlation matrix, Poisson error distribution and a log link function to model the number of pollen grains per slide as a function of pollination system, distance from the male inflorescence and wind speed. Each 10 minute run was treated as the subject to account for non-independence. Pollination system and species were considered factors, with distance and wind speed as covariates. We accounted for the variation in the amount of pollen available for each male inflorescence by using the log of pollen grain availability, which was estimated as the mean number of pollen grains per flower for that species multiplied by the number of open flowers, as an offset (a structural predictor with a fixed coefficient of one). Fixed predictors in this model were pollination system, distance, wind speed, species nested within pollination system and interactions between pollination system, distance and wind speed. Score statistics were used to assess significance.

FLORAL TRAITS

Inflorescence measurements

We measured the height (h) and width (w) of 20 male and 20 female inflorescences per species. We determined inflorescence size by calculating its surface area to acquire a single value for inflorescence size, and used the area of a cylinder (2π [½w] (h+ [½w])) since this shape was the closest to that of most *Leucadendron* inflorescence. For each inflorescence measured, we counted the number of flowers per inflorescence for both sex. We established the number of inflorescences per branch (inflorescence density) by counting the number of inflorescence per 1cm width diameter branch for 20 male and 20 female plants for all species except *L. argenteum* where five male and five female plants were sampled (due to difficulties in sampling the tree > 6m tall). A branch's diameter was measured progressively until a standardized 1cm width apparatus fitted the branch, then all the inflorescences above the 1cm width mark were counted. We decided on a standardized measurement of 1cm width diameter branch size, to account for consistent amount of resources travelling to the inflorescence and which would enable standard comparisons among the *Leucadendron* species studied.

Pollen

We determined pollen production per flower by estimating the number of pollen grains in five pre-dehiscent male flowers per species. Each flower was placed in a separate Eppendorf vial with 70% ethanol, which was vortexed for three minutes to break open the anthers. We added fuchsin-stained glycerol, which assisted in suspending pollen grains in the solution, up to a standardized volume of 1 ml and vortexed for a further two minutes to achieve homogenous pollen grain dispersal. We counted pollen grains in five subsamples of 2 μl from each sample and extrapolated total pollen production per flower from the initial sample volume of 1 ml. We then calculated the amount of pollen produced per male inflorescence (pollen grains per flower × number of flowers per inflorescence) and subsequently the amount of pollen per branch (amount of pollen produced per inflorescence × number of inflorescences per branch).

We measured pollen dimensions by imaging pollen grains with a Scanning Electron Microscope (Zeiss Evo LS 15; Fig. S2, S3) and using AnalySis ® (version 3.2) on the SEM photographs with the scale bar as reference. We measured the vertical height (h) and base (b) of 15 triangular pollen grains (Fig. S2, S3) from at least 15 anthers for each species and calculated the pollen size by using the surface area equation of a triangle (½ × b × h), to acquire a single value for pollen size.

Subtending leaf colour

We measured the spectral reflectance of the subtending leaves surrounding inflorescences (one leaf per plant for eight plants) for both sexes per species over the waveband of 300-700nm using a reflectance spectrophotometer (Ocean Optics In. Dubebin, Fla. – see details in Johnson and Andersson, 2002). Furthermore, one green leaf further down from the inflorescences on the same plant was sampled to represent the background colour. Colour reflectance patterns of the subtending leaves and green leaf background were plotted in a two-dimensional colour space using segment classification analysis (Endler 1990). We used this approach because vision models for the beetle pollinators of *Leucadendron* are not available, and segment classification provides a rough assessment of how colour is perceived by animals with an opponency visual system (i.e. one that allows animals to detect colour from the differences in responses of pairs of receptors that are sensitive to different wavelengths).

Following Endler (1990), we determined colour contrast (Euclidian distance), by calculating the colour score (x; y) for the green leaves for each plant per species. We then subtracted from the x and y values of the subtending leaves from each green leaf colour score. Euclidian distance was calculated and averaged, resulting in one value for male and female leaves per *Leucadendron* species. A Euclidian distance of zero signifies no colour contrast to the green leaf background, and increasing distance values represent increasing contrast to the background. Differences in colour contrast between wind- and insect-pollinated species were assessed using phylogenetic generalized estimating equations (pGEEs, see below).

Floral scent sampling

We used dynamic headspace scent extraction from male and female inflorescences of the 17 *Leucadendron* species. We sampled four inflorescences of male and female inflorescences, respectively, for each species, accompanied by two *in situ* control samples of leaf material and ambient air. Before taking headspace samples, we counted the number of inflorescences to be sampled. Each sample was enclosed in a polyacetate oven bag through which an absorbent tube cartridge containing a mixture of 1.5 mg Tenax-TA® and Carbotrap® (held between glass wool plugs) was inserted with air pumped at a flow rate of 200 ml min^{-1} for 15 minutes. We thermally desorbed the samples in a Varian CP – 3800 gas chromatograph (Varian, Palo, Alto, California) following the protocol of Shuttleworth and Johnson (2009).

We used Varian Workstation software with NIST 11 MS search software 2.0 to identify compounds and used retention times for comparisons of published Kovats retention indices. Compounds present in the inflorescences samples with similar abundance to those in the controls were deemed to be contaminants and excluded from analysis. However, compounds in the inflorescence samples that were more than double the peak area in the controls, were included by subtracting the peak area of the control sample from the inflorescence sample. We quantified emission rates per flower and inflorescence per hour by injecting standard amounts of methyl benzoate into an absorbent tube cartridge, which were thermally desorbed as per the methods above. Comparisons of the number of volatile compounds and emission rate between wind- and insect-pollinated species involved pGEEs (see below).

PHYLOGENETIC ANALYSES

Phylogenetic data

We used the Maximum Clade Credibility chronogram (MCC chronogram) from a sample of 1000 chronograms for analyses, which were extracted by sampling every 5000th generation from Bayesian Markov Monte Carlo Chain (MCMC) run in BEAST after excluding the first 10% (i.e. 5 million generations) burn-in to ensure only stationary distributions were sampled (Hoffmann 2012).

Our analyses considered pollination system (wind/insect) as a discrete trait. Continuous traits under consideration were (A) for male inflorescences: pollen size (μm^2), amount of pollen per (1) flower, (2) inflorescence, and (3) branch, and (B) for both male and female inflorescences: number of florets per inflorescences, size of inflorescence (mm^2), number of inflorescences per branch, colour contrast (Euclidian distance) between subtending and green background leaves, number of volatile compounds per inflorescence, scent emission rate per flower (ng flower^{-1} h^{-1}) and inflorescence (ng inflorescence^{-1} h^{-1}) and degree of sexual dimorphism for each of traits.

Because pGEEs use only species averages, we calculated an index of sexual dimorphism derived from Harris and Pannell (2010) for each floral trait and each species:

$$\text{Sexual dimorphism} = = 2\ \frac{X_f - X_m}{X_f + X_m}$$

Where X_f and X_m are species averages for females and males, respectively, for the trait under consideration. Female mean floral traits were often greater than male mean floral traits. According to Harris and Pannell (2010), this index for the degree of sexual dimorphism controls for any allometric relationship between sexual dimorphism and mean floral traits because effectively it is the absolute difference in floral traits between sexes divided by the mean floral trait. Thus, we used the absolute difference when negative values were present. Consequently, an index of sexual dimorphism score of zero indicates no difference in the floral trait between males and females, and scores further from zero indicates higher levels of sexual dimorphism.

Phylogenetic signal

We examined the degree of phylogenetic signal in pollination system of *Leucadendron* species and for all examined floral traits. For discrete traits, we compared the number of steps needed for parsimony reconstruction over the pruned MCC chromogram to the number of steps required if the same character is randomly re-shuffled 1000 times in Mesquite (version 2.75; Maddison and Maddison 2011), whilst ensuring that the proportion of states is kept constant. If the number of steps in the observed state distribution lies outside the 95% confidence interval of the randomized state distribution, the hypothesis of a phylogenetically random distribution is rejected and significant phylogenetic influence on trait occurrence is assumed.

For continuous traits, we used the function ‘phylosignal’ in the R (version 2.15) package ‘picante’ (Kembel et al. 2010), to calculate the *K* statistic (Blomberg et al. 2003) and to assess the probability related to the comparison of the variance of phylogenetically independent contrasts between the 1000 randomized trait distributions and the observed trait distributions over the MCC chronogram. We accounted for phylogenetic uncertainty by estimating K for 1000 chronograms. Because of non-normality of the resulting *P* value distributions, we use the median *P* value to determine whether trait magnitude is significantly influenced by phylogenetic structure.

Ancestral state reconstruction

To examine the evolutionary history of pollination systems we conducted ancestral state reconstruction using parsimony methods as implemented in Mesquite. Trait distribution over the pruned trees used in our analyses rendered Maximum Likelihood (ML) methods unable to unequivocally reconstruct trait evolution, whereas parsimony methods yielded unequivocal reconstructions for most nodes. We used parsimony reconstruction to determine optimal states for all internal node in a sample of 1000 chronograms, and summarize this information on the MCC chronogram. Nodes were interpreted as transitional if the state considered optimal in ≥ 75% of trees varied at older nodes from the state considered optimal.

Phylogenetic generalized estimating equations (pGEEs)

We analysed differences in quantitative floral traits between wind- and insect-pollinated species using generalized linear models coupled with generalized estimating equations (GEEs) that account for non-independence among species due to phylogenetic relatedness (Paradis and Claude 2002) as implemented in the R package ‘ape’ (version 3.0–2; Paradis et al. 2004). Continuous dependent variables were analyzed with either a Poisson error distribution and log link function, or a normal distribution (in some cases log transformed) and identity link function. We accounted for phylogenetic uncertainty by repeating each analysis for 1000 chronograms.

Results

POLLEN MOTILITY IN A WIND TUNNEL

Pollen mobility of wind-pollinated *Leucadendron* species differed significantly from that of insect-pollinated species ($\chi^2 = 26.28$, df = 1, $P < 0.0001$; Fig. 3). The mobility of pollen grains for both insect- and wind-pollinated was significantly greater at higher wind speeds ($\chi^2 = 60.44$, df = 1, $P < 0.0001$). There was a highly significant effect of pollination system ($\chi^2 = 40.59$, df = 4, $P < 0.0001$) and a significant interaction between pollination system, distance and wind speed ($\chi^2 = 24.57$, df = 2, $P < 0.0001$; Fig. 3).

FLORAL TRAITS

Subtending leaf colour

In insect-pollinated *Leucadendron* species the subtending leaves surrounding the inflorescence differed strongly from the green leaf background in brightness and chroma, largely due to the showy subtending leaves demonstrating strong reflectance between 570 – 590 nm (yellow wavelength) (Fig. S4). Male subtending showy leaves of insect-pollinated species also often had greater spectral reflectance than those surrounding female inflorescences (Fig. S4). By comparison, mean spectral reflectance of the subtending leaves of wind-pollinated species were very similar to that of the green leaf background (Fig. S5).

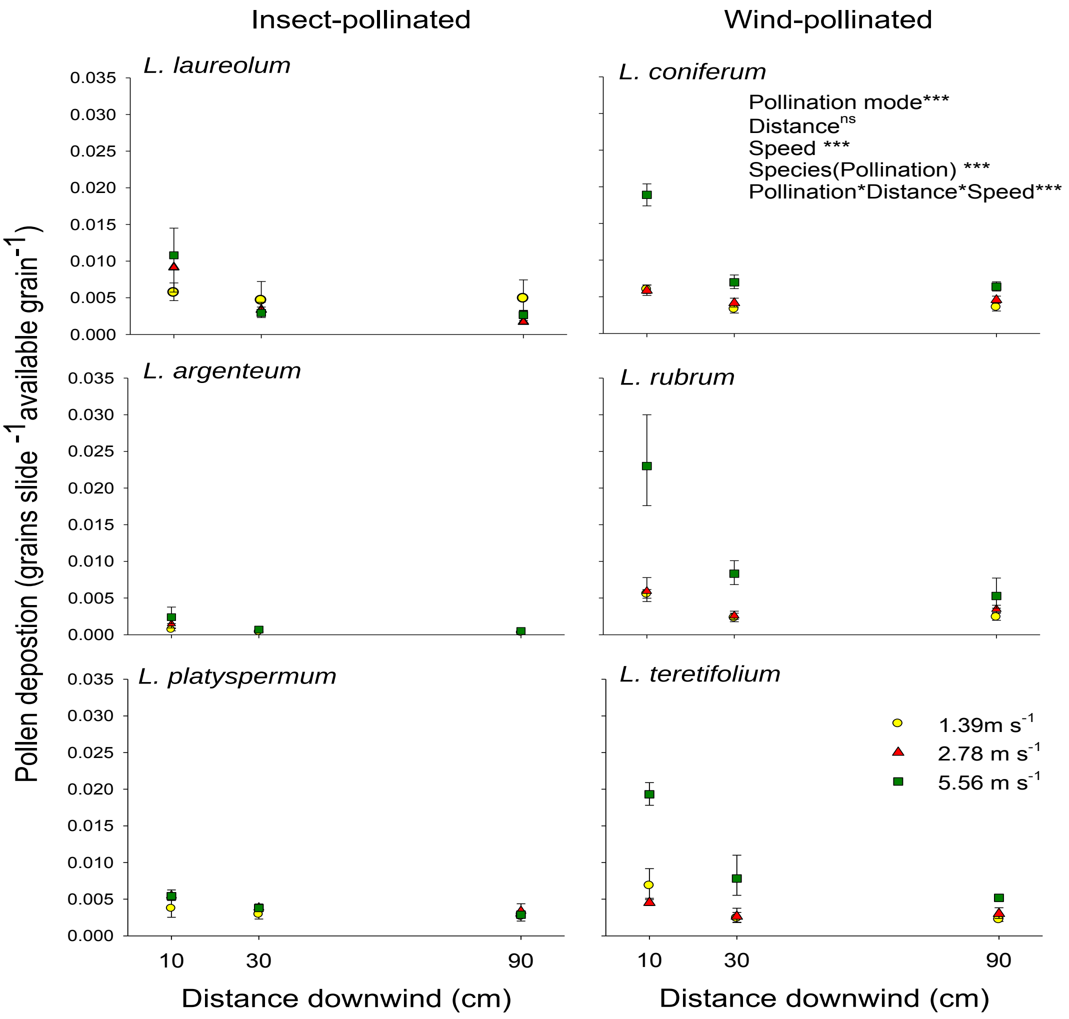

Figure 3. Mean (± SE) pollen deposition (grains slide^{-1} available grain $^{-1}$) on sticky fuchsin-gel coated microscope slide placed downwind of a male inflorescence at three separate wind speeds (1.39 m s^{-1}, 2.78 m s^{-1} and 5.56 m s^{-1}) in a wind tunnel. In three clades wind- versus insect-pollinated species (*L. coniferum* vs. *L. laureolum*, *L. teretifolium* vs. *L. platyspermum* and *L. rubrum* vs. *L. argenteum*) were compared for pollen motility. Significance values are given for the main effects and their interaction in a GEE analysis. ^{ns} $P > 0.05$, *** $P < 0.001$

Plotting of these values into a two-dimensional colour space using Endler's (1990) segment classification indicated that the subtending leaves of both male and female plants of wind-pollinated species fell mainly among the values for the green leaf background (Fig. 4), thus indicating negligible difference in colour contrast between them. In the case of insect-pollinated species, the showy subtending leaves of both male and female plants mostly occupied an independent colour space from wind-pollinated species and the green leaf background, indicating a distinct colour contrast. The main exception to this trend is the insect-pollinated *L. argenteum* (the silver tree), whose subtending leaves fall among the green leaf background. The colour scores of showy subtending leaves of male inflorescences of insect-pollinated *Leucadendron* species were at the greatest angle from the origin and therefore have the greatest hue, followed by showy subtending leaves of female inflorescences of insect-pollinated *Leucadendron* species, subtending leaves of the inflorescence of wind-pollinated *Leucadendron* species and finally the green leaf background.

After adjusting for phylogenetic relatedness, colour contrast between subtending and green leaves differed significantly only between male inflorescences, with contrast being significantly stronger in insect- than in wind-pollinated species. (Fig. 7D). Additionally, wind-pollinated species had a significantly higher degree of sexual dimorphism than insect-pollinated species in colour contrast (Fig. 7D).

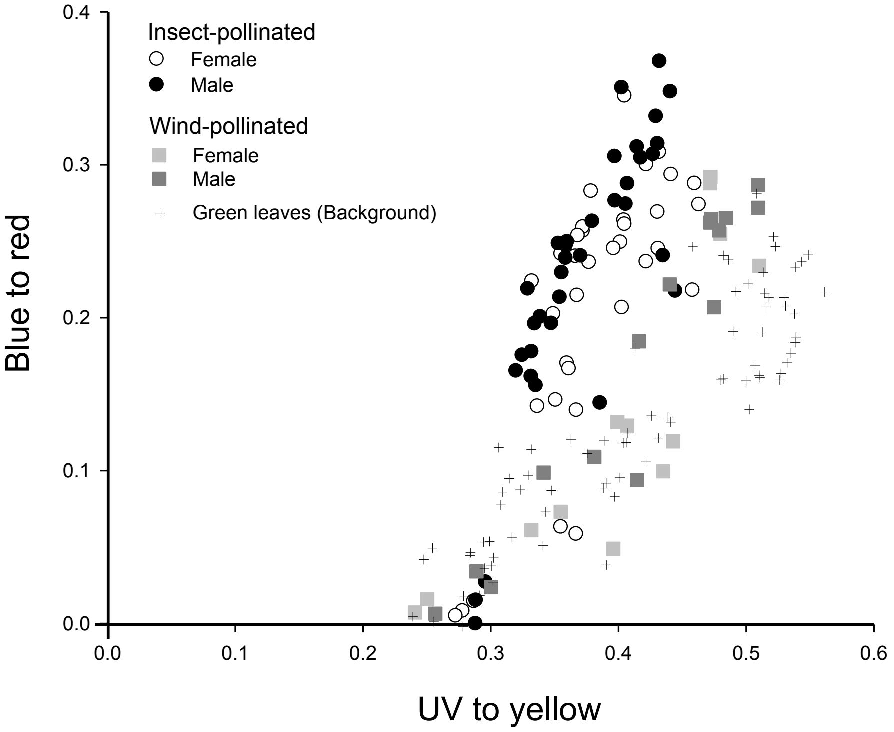

Figure 4. Reflectance color scores of green leaf background and the subtending leaves surrounding male and female inflorescences of insect- and wind-pollinated *Leucadendron* species plotted in Endler’s (1990) color space.

Floral scent sampling

We detected 111 volatile compounds in the floral scent of the examined *Leucadendron* species, and were able to identify 88 compounds (79%, Table S3). The identified compounds included a large variety of volatile compounds, including aliphatic alcohols (7), aliphatic aldehydes (4), aliphatic esters (14), alaphatic ketones (5), benzenoides and phenylpropanoids (19), monoterpenoids (25), sesquiterpenoids (10), sulfur-containing compounds (1), nitrogen-containing compounds (3) (Table S3).

Compounds that occurred consistently in both sexes of insect-pollinated species were methylbenzoate, linalool, caryophylene, m/z: 150*, 69, 41, 81, 53, 79, 107, 39, 135, 82, 67 (Table S3). *L. sessile* and *L. tinctum* have the greatest number of emitted floral compounds for both male (29±5.2 and 29±0.5, respectively) and female (28±2.6 and 35±0.5, respectively) inflorescences. Hex-4-en-1-yl acetate was among the most abundant compound in both species and both sexes' inflorescences. Furthermore *L. tinctum* and *L. sessile,* as well as *L. platyspermum,* and *L. spissifolium* subsp. *spissifolium* had the greatest emission rate per flower and inflorescence for both sexes (Table S3).

In wind-pollinated species, linalool occurred consistently in both sexes of all species (Table S3). Caryophyllene was present in male inflorescences of all wind-pollinated species. In *L. salicifolium,* caryophyllene was one of the most abundant compounds and occurred in both sexes (Table S3). (E)-Ocimene was present in all wind-pollinated species except for *L. dubium*. Sabinene was present in four wind-pollinated species and mostly emitted by female inflorescences, and constituted >40% of the floral scent composition in female inflorescences of *L. coniferum, L. dubium* and *L. rubrum* (Table S3).

After adjusting for phylogenetic relatedness, insect-pollinated species of both sexes emitted more floral volatile compounds (Fig. 7E) than wind-pollinated species, and male inflorescences of insect-pollinated species emitted significantly more floral volatile compounds than those of wind-pollinated species. Wind-pollinated species had significantly greater sexual differences in scent composition than did insect-pollinated species, although the overall degree of sexual differences was low (Fig. 7E).

Flowers and inflorescences of both sexes of insect-pollinated species emitted floral volatiles at a significantly greater rate (ng hr^{-1}) than those of wind-pollinated species (Fig. 7F,G). However, sexual differences in floral volatile emission rate per flower and per inflorescence was stronger in wind- than in insect-pollinated species (Fig. 7F,G).

Furthermore, floral volatile emission rate per flower exhibited a high degree of sexual differences for both pollination systems (Fig. 7F), whereas, only wind-pollinated species exhibited a high degree of sexual differences in floral volatile emission rates per inflorescence (Fig. 7G).

PHYLOGENETIC ANALYSES

Among the floral traits measured, only pollen size (μm²) and the colour contrast between the subtending leaves and the green background leaves exhibited significant phylogenetic signal (Table 1). Parsimony analysis identified the root node as insect-pollinated (Fig. 5) and wind-pollination as a derived trait that evolved between four and five times in the genus (median estimate = 5 and mean estimate = 4.583 over 1000 chronograms, 17 species; Fig. 5).

Table 1. Phylogenetic signal of floral traits in *Leucadendron*. (A) Discrete trait: phylogenetic conservatism is shown whether the number of parsimony steps in observed state distribution is greater than 95% confidence interval (UCI, upper confidence interval; LCI, lower confidence interval) Over 1000 trait reshuffles, accounting the mean (LCI – UCI). (B) Continuous traits: P-value is indicated as the probability of a certain K due only to sampling error. K is accounted as mean ± SE and P as the median (1st quartile, 3rd quartile) because of non-normality. n (number of sampled species) = 17

		Parsimony steps in observed state distribution	Parsimony steps in randomized state distribution
(A)	Discrete traits		
	Pollination	5 (4,5)	5 (5,5)
(B)	Continuous traits	K	P
	Pollen size (μm^2)	0.64 ± 0.007	**0.02** (0, 0.07)
	Pollen per flower	0.25 ± 0.004	0.80 (0.71, 0.90)
	Pollen per inflorescence	0.33 ± 0.004	0.52 (0.39, 0.64)
	Pollen per branch	0.43 ± 0.003	0.26 (0.20, 0.33)
	Inflorescence size (mm^2)		
	Male	0.37 ± 0.004	0.39 (0.21, 0.55)
	Female	0.49 ± 0.005	0.15 (0.04, 0.27)
	Flowers per inflorescence		
	Male	0.40 ± 0.003	0.33 (0.21, 0.46)
	Female	0.39 ± 0.004	0.34 (0.25, 0.43)
	Inflorescence density		

Male	0.38 ± 0.003	0.32 (0.21, 0.43)
Female	0.40 ± 0.003	0.33 (0.18, 0.47)
Colour contrast between subtending and green background leaves		
Male	0.47 ± 0.006	0.13 (0.002, 0.26)
Female	0.70 ± 0.005	**0.02** (0.002, 0.03)
Inflorescence volatile compounds		
Male	0.39 ± 0.005	0.33 (0.17, 0.49)
Female	0.25 ± 0.004	0.73 (0.01, 0.80)
Emission rate per flower (ng h^{-1})		
Male	0.43 ± 0.005	0.27 (0.14, 0.39)
Female	0.31 ± 0.004	0.59 (0.46, 0.73)
Emission rate per inflorescence (ng h^{-1})		
Male	0.34 ± 0.004	0.49 (0.39, 0.60)
Female	0.33 ± 0.004	0.53 (0.39, 0.67)

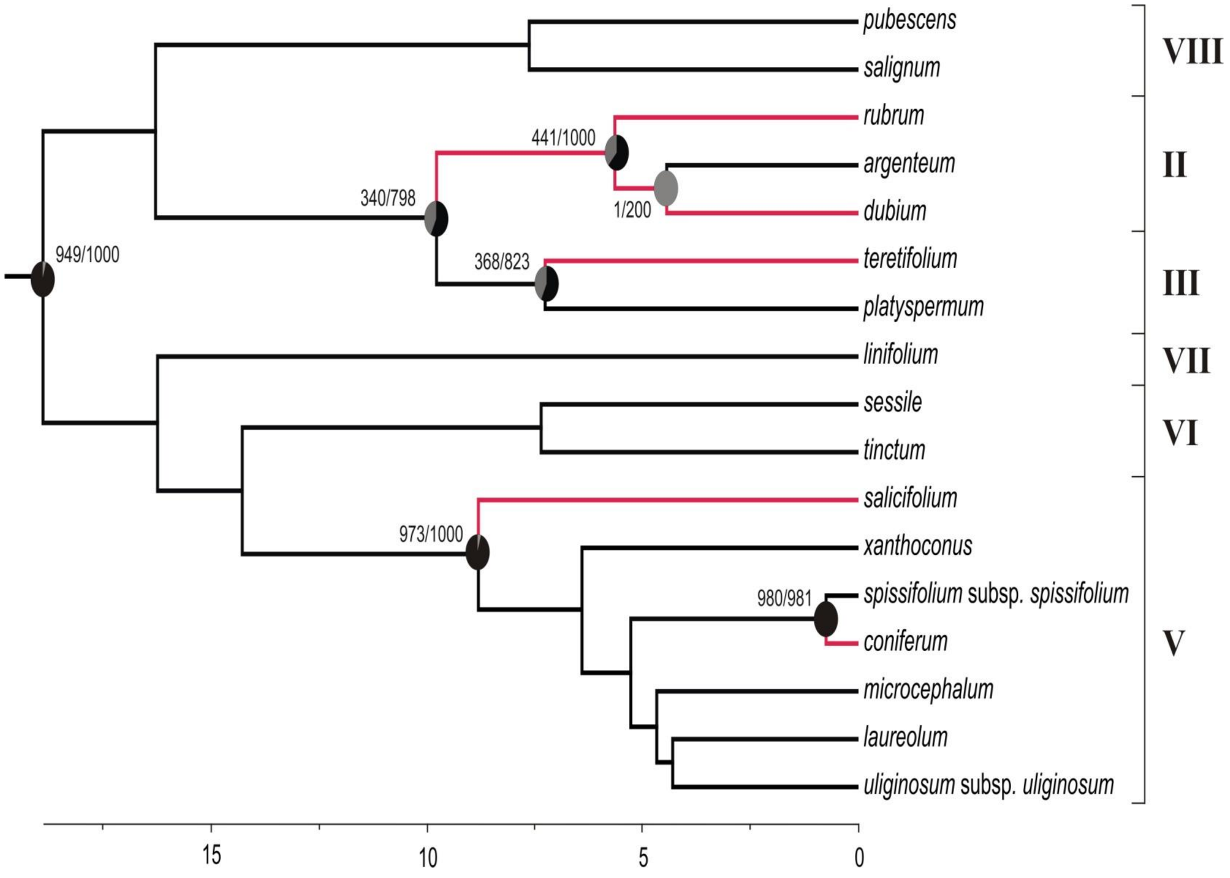

Figure 5. Parsimony reconstruction of the repeated (≥ 4 x) evolution of wind pollination (red branches) from insect-pollinated (black branches) ancestors in 17 *Leucadendron* species. Parsimony reconstructions of ancestral states over 1000 chronograms are summarized on the Bayesian maximum clade-credibility chronogram (Hoffmann 2012). Node support values are only shown for nodes with uncertainty (black: insect pollination, grey: uncertainty), all other nodes resolved as 100% insect pollination. Support values represent the number of trees for which the state was determined as optimal / number of trees with the node. For support of all nodes see Table S2 and Fig. S6**.** Rectangular brackets delimit *Leucadendron* clades *sensu* Barker et al. (2004).

Traits associated only with male inflorescences - Wind-pollinated species had significantly smaller pollen grains than insect-pollinated species, after adjusting for phylogenetic relatedness (Fig. 6A). Wind-pollinated species also had smoother, more uniformly shaped pollen grains compared to insect-pollinated species (Fig. S2, S3). The amount of pollen grains produced per inflorescence did not differ significantly between insect- and wind- pollinated species (Fig. 6C), however, wind-pollinated species produced significantly greater amounts of pollen grains per flower and per branch compared to insect-pollinated species (Fig. 6B,D).

Traits associated with male and female inflorescences – After adjusting for phylogenetic relatedness, there was no significant difference in inflorescence size between the insect- and wind-pollinated species for either sex (Fig. 7A). Insect- and wind-pollinated species did also not differ significantly in their degree of sexual dimorphism, which was low for both pollination systems; although insect-pollinated species did have slightly greater degree of sexual dimorphism then wind-pollinated species (Fig. 7A). Insect- and wind-pollinated species did also not differ significantly in the number of flowers per male or female inflorescence. Sexual dimorphism of flower number was pronounced in both insect- and wind-pollinated species, but did not differ significantly amongst pollination systems (Fig. 7B).

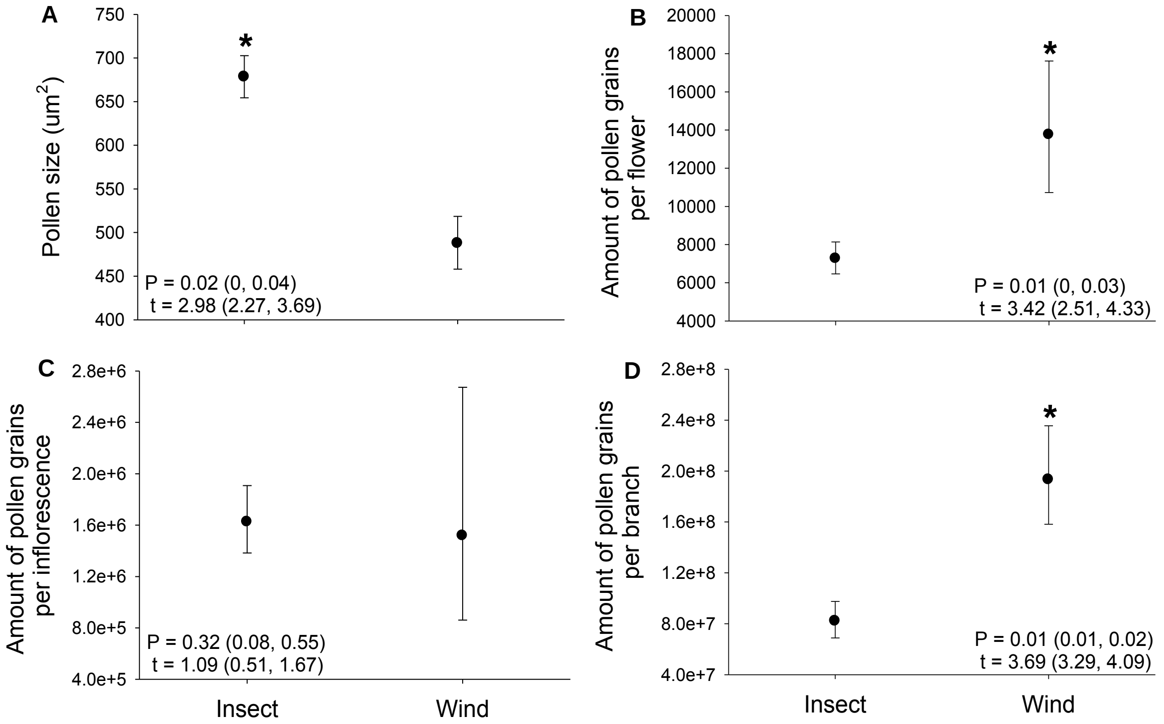

Figure 6. Comparison of floral traits associated with only male inflorescences between wind- and insect-pollinated *Leucadendron* species using phylogenetic estimating equations (pGEEs) and accounting for phylogenetic uncertainty by repeating analyses for 1000 chronograms. The corresponding 1000 probability values and t statistics are summarized as medians (1st quartile, 3rd quartile) because of non-normality of P- and t-value distributions. Degrees of freedom = 7.7, number of species = 17 for all traits. * $P < 0.05$.

Wind-pollinated species had significantly more male and female inflorescences per branch than insect-pollinated species (Fig. 7C). The number of male inflorescences per branch was almost tenfold greater than that of female inflorescences in both insect- and wind-pollinated species. Correspondingly, we found a high degree of sexual dimorphism between the sexes, but no significant difference in sexual dimorphism amongst pollination systems (Fig. 7C).

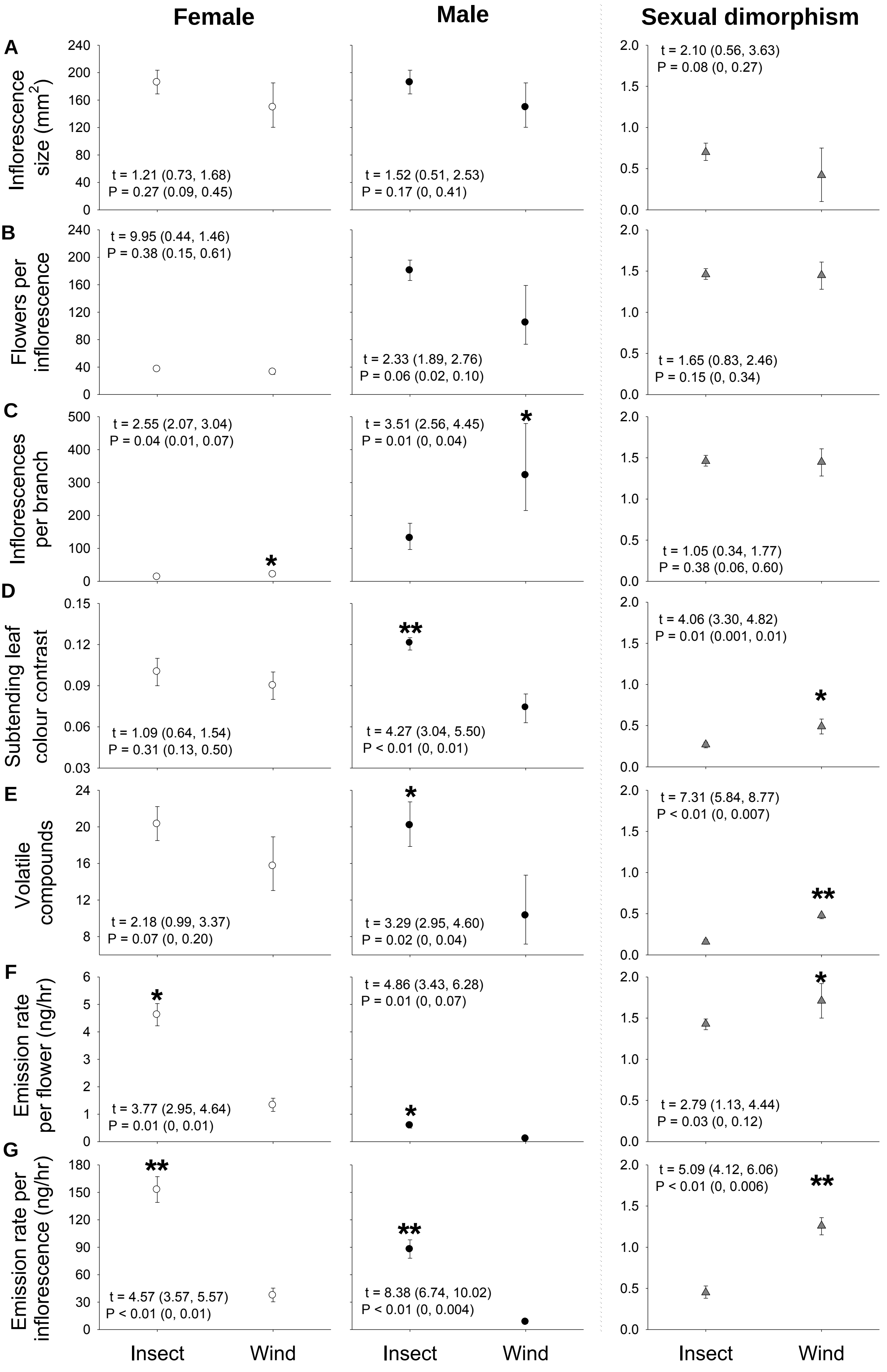

Figure 7. Comparison of floral traits of male and female inflorescences between wind- and insect-pollinated *Leucadendron* species, as well as an index of sexual dimorphism between

the sexes, using phylogenetic estimating equations (pGEEs) accounting for phylogenetic uncertainty by using 1000 chronograms. P and t are medians (1st quartile, 3rd quartile) because of non-normality. d.f., degrees of freedom for the pGEE t-test is 7.7 and n (the number of sampled species) is 17, for all traits respectively. * $P < 0.05$, ** $P < 0.01$

Discussion

Our analyses suggest that transitions from insect to wind pollination occurred at least four times during the diversification of *Leucadendron* (Fig. 5). By comparing floral traits of insect- and wind-pollinated *Leucadendron* species, we ascertained that several floral traits underwent evolutionary modification during these transitions.

The pollen of wind-pollinated *Leucadendron* species underwent key modification in terms of mobility, size and production. In a wind tunnel, we determined that pollen grains of wind-pollinated *Leucadendron* species had more motility than insect-pollinated species (Fig. 3). This could be attributed to the significantly smaller size of pollen grains of wind-pollinated species compared to those of insect-pollinated species (Fig. 6A). Similarly, among *Schiedea* species abundant and relatively small-size pollen grains are a key feature of wind-pollinated species which are otherwise morphologically similar to insect-pollinated species (Weller et al. 1998). According to Niklas (1985) the inertia of small pollen grains is low, thereby assisting effective removal from anthers and decreases settling velocity, which allows pollen grains to be transported a greater distance. Furthermore, pollen grains of wind-pollinated *Leucadendron* species had smoother, more uniformly shaped pollen grains than insect-pollinated species (see Fig. S2, S3), which could also aid in the greater mobility of pollen grains in airstreams and thus more motile pollen grains. Using detached male inflorescences in the wind tunnel might limit the full potential of pollen dispersal since we could not take into account the movement of the inflorescences on the entire plant. However, even though the wind tunnel might not represent an entirely natural situation, the standardization of experiment demonstrates the differences in the capability of the six *Leucadendron* species to disperse their pollen by wind.

Pollen production per flower was significantly greater in wind-pollinated species than in insect-pollinated species (Fig. 6B). Given that each female flower of *Leucadendron* species produces only one ovule (Williams 1972), the pollen:ovule ratio was hence also significantly greater for wind-pollinated species. Wind-pollinated *Leucadendron* species, therefore, follow a common pattern among wind-pollinated plants by having a single ovule per flower (Pohl 1929; Friedman and Barrett 2008) and a high pollen:ovule ratio (Whitehead 1969; Stebbins 1970; Faegri and van der Pijl 1979; Cruden 2000; Friedman and Barrett 2009). The reduction of ovules per flower in wind-pollinated plants is thought to generally evolve after wind pollination (Linder 1998) due to the improbability of numerous pollen grains landing on a stigma (Pohl 1929; Dowding 1987; Friedman and Barrett 2008). Given that, pollen grains of wind-pollinated species are usually transported singularly (i.e. no clumping) (Goodwillie 1999; Friedman and Barrett 2008). However, a single ovule is the ancestral condition in *Leucadendron* (Williams 1972) and should therefore be interpreted as a pre-adaptation rather than adaptation for wind-pollination.

Wind-pollinated species produced thousands more pollen grains per branch (1cm width) than did insect-pollinated species (Fig. 6D). We demonstrated that, in comparison to insect-pollinated species, wind-pollinated species have three-fold more male inflorescences per branch (Fig. 7C), thus clarifying the reason for prodigious pollen production per branch (Fig. 6D), especially given that per inflorescence pollen production did not differ between the two pollination systems (Fig. 6C). The result of having hundreds of male inflorescences is that a single shake of a branch scatters millions of pollen grains into the airstream for highly effective pollen dispersal (pers. obs. M. Welsford). Similarly, in the dioecious wind-pollinated Amazonia palm species, *Mauritia flexuosa* (Arecaceae), having numerous flowers and prodigious pollen production were morphological trait adaptations facilitating wind pollination (Rosa and Koptur 2013).

The requirement to capture pollen grains in the airstream for wind-pollinated species often results in changes to inflorescence structure and placement (Niklas 1987; Cox 1991; Culley et al. 2002). Female plants of wind-pollinated *Leucadendron* species have significantly more female inflorescences per branch than insect-pollinated species (Fig. 7C), which could aid in pollen capture by influencing the pattern of airflow to channel pollen to stigmas (Niklas 1985). Linder and Midgley (1996) showed that stigmas of the wind-pollinated species, *Leucadendron rubrum*, could effectively capture conspecific pollen grains in a community with four co-flowering wind-pollinated species. In addition, the stigmatic surface is larger in wind-pollinated *Leucadendron* species than it is in insect-pollinated species (Williams 1972) and this is considered to assist in pollen capture (Whitehead 1983; Niklas 1987; Friedman and Barrett 2011). In addition, whereas, insect-pollinated *Leucadendron* species often have leaves surrounding the inflorescence that extend past or even fully enclose female inflorescences (as exemplified by *L. laureolum*

(Fig. 1C)), wind-pollinated *Leucadendron* species have fully exposed inflorescences, which could facilitate airborne pollen receipt (Fig. 2) and the evolution of wind pollination in the genus. Furthermore, the flowers of *Leucadendron* are condensed into inflorescences which could aid in pollen receipt and dispersal in wind-pollinated species because of the harmonic oscillations created by condensed inflorescences (Niklas 1985, 1987). Indeed, Weller et al. (1998) found among wind-pollinated *Schiedea* species, a prominent characteristic is a tendency toward flowers being condensed into small inflorescences. Moreover, in *Leucadendron* the inflorescences are a single condensed unit in both pollination systems (Fig. 1, 2), thus dimorphism between the sexes probably does not influence the structure of inflorescences.

In terms of Endler's (1990) generalized colour space, we ascertained that insect-pollinated *Leucadendron* species have leaves which are yellower and showier than those of wind-pollinated *Leucadendron* species. Subtending leaves of the latter are similar to those of the green leaf background and therefore inconspicuous (Fig. 4). Furthermore, greater colour contrast between the showy subtending leaves and the green leaves was found in insect-pollinated *Leucadendron* species compared to wind-pollinated species for both sexes (Fig. 7D). These yellower and more conspicuous subtending leaves, of insect-pollinated *Leucadendron* species are probably a visual cue that plays a role in attracting pollinators. This is supported by the observation that these showy subtending leaves only turn to yellow from green during the flowering period (Schmeisser et al. 2010) and that the actual flowers of *Leucadendron* species are small, inconspicuous and often hidden by these subtending leaves (see Fig. 1,2). In addition, Hemborg and Bond (2005) found that by removing the showy subtending leaves around female inflorescences of *L. xanthoconus*, fewer *P. cinerascens* landed on the exposed inflorescences, further highlighting the importance of these leaves as visual cues and possible shelter for the beetles. Moreover, the significance of the showy subtending leaves as visual cues to attract pollinators is apparently crucial, given that we found other inflorescence traits such as the number of flowers and size of inflorescences did not differ significantly between wind-and insect-pollinated species (Fig. 7D-G).

Floral scent has been shown to play an important role in pollination systems shifts (see Raguso and Pichersky 1995; Micheneau et al. 2006; Muchhala, 2006; Muchhalla and Thomson 2010; Shuttleworth and Johnson 2010; Wragg and Johnson 2011; Steenhuisen et al. 2013). We determined that floral scent plays an important role in the evolutionary transition to wind pollination in *Leucadendron*, given that floral scent emission and the number of volatile compounds detected in the scent of insect-pollinated species were significantly greater than that recorded from wind-pollinated species (Fig. 7E-G). Wragg and Johnson (2011) also found that in sedges insect-pollinated species had greater floral emission rates than wind-pollinated species, which is a critical aspect in the shift from wind-to insect pollination. The low floral scent production by wind-pollinated species is probably due to them not requiring floral scent to attract pollinators. Interestingly, however, not all wind-pollinated species have low floral scent production (Jürgens et al. 2012). Jürgens et al. (2012) found that a wind-pollinated *Schiedea* species, *S. adamantis*, has a floral scent emission rate that is relatively high and similar to that of insect-pollinated *Schiedea* species. The floral scents of both insect- and wind-pollinated *Leucadendron* species are dominated by monoterpenes, benzenoides and phenylpropanoids and aliphatic esters. Insect-pollinated species were consistently found to emit methyl benzoate, linalool, caryophylene and an unknown compound (m/z: 150*, 69, 41, 81, 53, 79, 107, 39, 135, 82, 67), across all species and sexes (Table S3). Insect-pollinated *Leucadendron* species are generally pollinated by beetles, particularly *P. cinerascens* (Nitidulidae) (Hattingh and Giliomee 1989; Welsford et al. 2014). The floral scent emitted by beetle-pollinated species are often strong, reminiscent of rotting or ripe fruit, and occasionally aromatically spicy (Proctor et al. 1996; Gottsberger 1999; Proches and Johnson 2009; Steenhuisen et al. 2010), which is similar to the floral scent of insect-pollinated *Leucadendron* species being described as faintly sweet or lemony, fruity or strong spicy or yeasty (Williams 1972; Rebelo 1995). It is thought that beetle-pollinated flowers mimic the scent of fruit, because fruits emit aliphatic esters that are often found in the composition of the floral scent of beetle-pollinated flowers (Thien et al. 1975; Jürgens et al. 2000; Steenhuisen et al. 2010). While, insect-pollinated *Leucadendron* species floral scent was dominant in monoterpenes, numerous “fruity” esters were also detected, which is consistent with beetle pollination. Paradoxically, however, fleshy fruits are almost entirely absent in the fynbos (Manning 2008) and the Proteaceae is dominated by nut type fruits as an adaptation to safely store nutrient-rich seeds in nutrient-poor, fire-prone environment (Rebelo 1995). Consequently, it is unclear whether the fynbos insect *P. cinerascens* is typical of other nitidulid beetles which are attracted to fruit smells.

Insect-pollinated species tend to have larger inflorescences with more flowers (Fig. 7A,B) which might contribute to their attractiveness to pollinators. For instance, *L. tinctum* and *L. sessile,* both have large inflorescences with many flowers, especially the male inflorescences (see Fig. 1H,I), and are visited by hundreds of insects (Hattingh and

Giliomee 1989; Welsford et al. 2014). However, the overall number of flowers and size of inflorescences did not differ significantly between wind-and insect-pollinated species, suggesting that other floral traits such as visual and olfactory cues probably play the greatest role in attracting pollinators for these species (Fig. 7D-G).

We determined that the degree of sexual dimorphism did not differ significantly between wind- and insect-pollinated species with respect to inflorescence features, such as inflorescence size, number of flowers per inflorescence, and inflorescence density (Fig. 7A-C). However, the degree of sexual dimorphism for flowers per inflorescence and inflorescence density was high for both pollination systems, due to males producing greater numbers of flowers and inflorescences than females (Fig. 7B,C). Conversely, in wind-pollinated *Schiedea* species, female inflorescences contain more flowers than males, resulting in a distinct divergence in inflorescence condensation between the sexes (Weller et al. 1998, 2007). These results are consistent with Bond and Midgley (1988) and Bond and Maze (1999) suggestion that selection acting on males to maximize their siring success, by producing more inflorescences, has in part shaped sexual dimorphism in *Leucadendron*. Interestingly, the actual costs of insect versus wind pollination for males could be similar, given that wind-pollinated species have many small inflorescences and many small pollen grains, while insect-pollinated ones have fewer large inflorescences, and fewer larger pollen grains (Fig. 6, 7A,C). In terms of colour and scent, wind-pollinated species are clearly more sexually dimorphic than insect-pollinated species (Fig. 7D-G). Insect-pollinated species probably require less sexual dimorphism because morphological divergence could change the behaviour of pollinators and hamper effective cross-pollination (Vamosi and Otto 2002; Friedman and Barrett 2009). Female insect-pollinated *Leucadendron* plants offer no pollen rewards or detectable amounts of nectar (M.Welsford pers. obs.), therefore reduced sexual dimorphism in insect-pollinated species might reflect selection for females to closely resemble the male advertising signals**.** For instance, Ashman (2009) reviewed patterns of floral scent in sexually dimorphic plants and found that in species with rewardless females, sexual differences in floral scent were generally less common (i.e. female plants might need to smell like male plants scent to attract pollinators).

In conclusion, this study showed that the evolution of wind-pollination in *Leucadendron* was accompanied by modifications of several traits, notably pollen size and morphology which influence pollen motility in wind, inflorescence architecture and dimorphism, spectral reflectance of subtending leaves and the amount and composition of volatile emissions. These results offer key insights into the modification of floral traits involved in the transition from insect to wind pollination. There is scope for broadening the study to include more species and to address hypotheses relating to the role of population density, pollinator limitation, and scramble competition among males in mediating the selective advantages of wind versus insect pollination. It is also critical to better understand the cues that attract pollinators, particularly nitidulid beetles, to inflorescences of insect-pollinated *Leucadendron* species by employing colour and/or scent choices in the field and laboratory.

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CHAPTER 3

SUPPLEMENTARY MATERIAL

Table S1. Study sites and co-ordinates details of the 17 *Leucadendron* species examined in this study grouped by clade (Barker et al. 2004)

Leucadendron species	Pollination	Clade	Study sites	Co-ordinates
argenteum (L.) R. Br.	Insect[b]	II	Table Mountain Nature Reserve	33°57’S, 18°27’E
dubium (H. Buek ex Meisn.) E. Phillips & Hutch	Wind[b]	II	Cederberg Wilderness Area	32°24’S, 19°11’E
rubrum Burm. f.	Wind[b]	II	Table Mountain Nature Reserve	33°57’S, 18°23’E
platyspermum R. Br.	Insect[b]	III	Stanford farmlands	34°27’S, 19°33’E
teretifolium (Andrews) I. Williams	Wind[b]	III	Caledon farmlands	34°18’S, 19°20’E
coniferum (L.) Meisn	Wind[a,b]	V	Cape Point Nature Reserve	34°18’S, 18°26’E
laureolum (Lam.) Fourc.	Insect[b]	V	Silvermine Nature Reserve	34°05’S, 18°24’E
microcephalum (Gand.) Gand. & Schinz	Insect[b]	V	Hottentots Holland Nature Reserve	34°05’S, 19°03’E
salicifolium (Salisb.) I. Williams	Wind[b]	V	Hottentots Holland Nature Reserve	34°08’S, 18°56’E
spissifolium (Salisb. ex Knight) *I. Williams subsp. spissifolium*	Insect[b]	V	Limietberg Nature Reserve	33°37’S, 19°06’E
uliginosum R. Br. subsp. *uliginosum*	Insect[b]	V	Witfontein Nature Reserve	33°52’S, 22°24’E
xanthoconus (Kuntze) K.Schum	Insect[b]	V	Silvermine Nature Reserve	34°05’S, 18°24’E
sessile R. Br.	Insect[a,b]	VI	Elandskloof Nature Reserve	34°08’S, 18°55’E
tinctum I. Williams	Insect[a]	VI	Hottentots Holland Nature Reserve	34°13’S, 19°10’E
linifolium (Jacq.) R. Br.	Insect[b]	VII	Stanford farmlands	34°25’S, 19°30’E
pubescens R.Br.	Insect[b]	VIII	Sawadee - Cederberg farm	32°20’S, 18°59’E
salignum P.J. Bergius	Insect[a]	VIII	Fernkloof Nature Reserve	34°23’S, 19°15'E

[a] Hattingh and Giliomee 1989, [b] Welsford et al. 2014

Table S2. Reconstructed states from parsimony analyses of pollination systems in 17 *Leucadendron* species. A summarized representing each tree with a certain node at the state which was reconstructed as distinctively best for the identified node, and the number of trees that are distinctively best state which arose. Reconstruction of pollination system: Optimal state: 0 = wind pollination, 1 = insect pollination.

Node	Number of trees containing nodes	Optimal state	Number of trees with optimal state
2	1000	1	949
3	349	1	349
4	385	1	385
7	798	1	340
8	1000	1	1
		0	411
11	823	1	368
13	200	0	1
16	522	1	522
18	1000	1	1000
21	981	1	980
25	405	1	405
26	113	1	113
29	172	1	172
30	994	1	991
31	1000	1	973
34	536	1	536

Table S3. Average relative amounts (%) of the floral scent compounds of 17 *Leucadendron* species of both male and female inflorescences. Compounds are ordered by compound class with increasing retention times. KRI = Kovat's retention index. Identification criteria of compounds: A = assessment of MS and verified retention times from published data, B = assessment of MS with published data, C = assessment of MS and verified retention times from authentic standards. * by a relative amount indicates that that compound was present in the leaf background control but was more than double the peak area in the sample than in the leaf background. tr = trace amounts (< 0.1% of total peak area). CAS # = CAS (Chemical Abstracts Service) registry number.

Clade				II						III				V	
				L. argenteum		*L. dubium*		*L. rubrum*		*L. platyspermum*		*L. teretifolium*		*L. coniferum*	
Pollination				Insect		Wind		Wind		Insect		Wind		Wind	
Compounds	CAS #	KRI	Criteria	F	M	F	M	F	M	F	M	F	M	F	M
Number of samples				4	4	4	4	4	4	4	4	4	4	3	3
Average number of compounds				14 ± 1.2	11 ± 2.5	18 ± 0.3	8 ± 0.6	15 ± 1.8	22 ± 0.5	18 ± 0.7	23 ± 0.8	28 ± 0.6	29 ± 1.7	9 ± 0.3	5 ± 1.3
Median of scent emission per flower (ng/h)				3.74	0.41	1.68	0.02	1.31	0.26	24.50	0.79	2.59	0.96	0.46	0.08
Median of scent emission per inflorescence (ng/h)				198.3	134.8	11.8	3.1	38.0	3.6	563.6	75.3	155.5	29.8	24.2	9.4
1. ALIPHATIC COMPOUNDS															
Alcohols															
n-Pentan-1-ol	71-41-0	1254	A	-	-	-	-	-	-	-	-	-	-	-	-
Heptan-2-ol	72237-36-6	1321	A	-	-	13.0 (4)	-	-	-	-	-	-	-	-	-
Hexan-1-ol	928-96-1	1352	C	-	-	6.9 (4)	20.3 (4)	-	-	-	-	-	-	-	-
(Z)-Hex-3-en-1-ol	3658-80-8	1381	A	-	-	-	-	-	-	-	-	0.8 (4)*	0.5 (4)*	-	-
(E)-Hex-2-en-1-ol	928-95-0	1423	A	-	-	-	-	1.7 (4)	3.0 (3)	-	1.2 (4)	1.5 (4)	1.3 (4)	-	-
Oct-1-en-3-ol	3391-86-4	1462	C	-	-	-	-	-	-	-	-	0.5 (4)	0.2 (3)	-	-
n-Heptan-1-ol	111-70-6	1453	A	-	-	1.8 (4)	-	-	-	-	-	-	0.2 (4)	-	-
Aldehydes															
Pentanal	110-62-3	1076	A	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-enal	6789-80-6	1193	A	-	-	-	-	-	-	-	-	-	-	-	-
Heptanal	111-71-7	1221	A	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Hex-2-enal	6728-26-3	1232	A	-	-	-	-	3.9 (4)	13.4 (4)	-	-	20.9 (4)	13.7 (4)	-	-
Esters															
Butyl acetate	123-86-4	1138	C	-	-	-	-	-	-	-	-	-	-	-	-
Methyl hexanoate	106-70-7	1221	A	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl caproate	123-66-0	1250	A	-	-	-	-	-	-	-	-	-	-	-	-
2-Heptyl acetate	5921-82-4	1288	A	-	-	-	-	-	-	-	-	-	-	-	-
Methyl (Z)-3-hexenoate	2396-78-3	1282	A	-	-	-	-	-	-	-	-	-	-	-	-
Hexyl acetate	124-92-7	1297	C	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 2-hexenoate	13894-63-8	1303	A	-	-	-	-	1.1 (2)	3.3 (4)	-	-	-	-	6.8 (3)	-
Isoamyl isovalerate	659-70-1	1305	B	-	-	-	-	-	-	-	-	-	-	-	-
Hex-4-en-1-yl acetate	10094-40-3	1325	B	-	-	1.5 (3)*	8.9 (4)*	4.7 (2)*	2.3 (3)*	0.9 (1)	5.8 (2)	-	-	-	-
(E)-Hex-2-en-1-yl acetate	111-27-3	1350	A	-	-	-	-	0.8 (3)	6.8 (4)	-	-	-	-	-	-
3-Cyclohexen-1-ol, acetate	10437-78-2	1449	B	-	-	-	-	-	-	-	-	-	-	-	-

Methyl 2-hydroxy-3-methylpentanoate	41654-19-7	1531	B	-	-	-	-	-	-	-	-	-	10.0 (4)	-	-
Benzyl formate	104-57-4	1709	A	-	-	-	-	-	-	-	-	0.2 (4)	0.2 (4)	-	-
3-hydroxy-2,4,4-trimethylpentyl 2-methyl-propanoate	74367-34-3	1884	B	-	-	-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 158*,57,85,98,103,129,41,70,56,55,74		1425		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 85,57,41,43,98,56,119,60,102,55		1446		-	-	-	-	0.3 (4)	0.8 (4)	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 55,83,101,95,70,96,43,41,39,71		1488		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 70,55,83,101,95,96,43,41,39,71		1536		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 57,91,41,71,85,43,108,79,32,39		1905		-	-	-	-	-	-	-	-	-	-	-	-
Ketones															
Heptan-2-one	110-43-0	1210	B	-	-	-	-	-	-	-	-	-	-	-	-
2-Octanone	111-13-7	1300	A	-	-	16.1 (3)	-	-	-	-	-	-	-	-	-
6,10-Dimethyl-5,9-undecadien-2-one	686-67-8	1870	B	-	-	-	-	-	-	-	-	-	-	-	-
cis-Jasmone	488-10-8	1967	A	-	-	-	-	-	-	-	-	0.1 (3)	tr (2)	-	-
2-Ethylcyclohexanone	4423-94-3	2114		-	-	-	-	-	0.7 (4)	0.2 (4)	0.3 (4)	tr (3)	-	-	-
2. BENZENOIDS AND PHENYLPROPANOIDS															
Benzyl methyl ether	538-86-3	1412	A	-	-	-	-	-	-	-	-	-	-	-	-
Benzaldehyde	100-52-7	1537	C	3.9 (4)*	4.9 (4)*	6.9 (4)*	-	-	-	-	-	19.7 (4)*	24.5 (4)*	-	-
Methylbenzoate	93-58-3	1634	C	18.0 (4)	35.3 (4)	-	-	-	-	0.3 (4)*	0.1 (1)*	-	-	-	-
Estragole	140-67-0	1688	A	-	-	-	-	-	-	-	-	-	-	-	-
1,2-Dimethoxybenzene (veratrole)	91-16-7	1676	A	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl acetate	140-11-4	1678	C	-	-	-	-	0.4 (3)	1.0 (4)	-	-	-	-	-	-
Methyl phenylacetate	101-41-7	1770	A	-	-	-	-	0.2 (2)*	0.1 (3)*	-	-	-	-	-	-
Methyl salicylate	119-36-8	1792	C	4.4 (4)	12.9 (4)	-	-	-	-	-	-	-	-	-	-
Phenylethyl acetate	103-45-7	1839	A	-	-	-	-	0.3 (2)	0.4 (4)	-	-	-	-	-	-
p-Cymen-8-ol	1197-01-9	1853	A	tr (1)	tr (1)	1.8 (4)	-	0.5 (4)	0.8 (4)	0.3 (4)*	0.2 (2)*	0.2 (4)	tr (1)	0.2 (2)	0.1 (1)
Guaiacol	90-05-1	1870	A	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol	100-51-6	1882	C	27.0 (4)*	20.0 (4)*	6.6 (4)*	2.1 (1)*	5.0 (4)	4.4 (4)	1.1 (4)	7.7 (3)	6.3 (4)*	12.1 (4)*	-	-
Phenylethyl alcohol	60-12-8	1920	A	tr (1)	0.1 (2)	1.5 (4)	-	1.0 (4)*	0.6 (4)*	-	0.2 (4)*	0.6 (4)*	0.3 (3)*	-	-
Phenol	108-95-2	2026	A	-	-	-	-	-	-	-	-	0.1 (2)*	0.3 (4)*	-	-
Methyleugenol	93-15-2	2026	A	-	-	-	-	-	-	-	-	-	-	-	-
4-Methoxybenzaldehyde	123-11-5	2037	B	-	-	-	-	-	-	-	-	-	-	-	-
p-Cresol	106-44-5	2098	A	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl tiglate	37526-88-8	2121	B	-	-	-	-	-	-	-	-	-	-	-	-
1,3,5-Trimethyoxybenzene	621-23-8	2151	B	-	-	-	-	-	-	-	-	-	-	-	-
3. TERPENOIDS															
Monoterpenes															
α-Pinene	80-56-8	1109	C	-	-	-	-	-	-	-	-	-	-	-	-
Sabinene	3387-41-5	1157	A	10.4 (4)	12.4 (4)	7.8 (4)	42.3 (4)	-	42.2 (4)	-	4.3 (4)	-	7.1 (4)	44.8 (3)	70.8 (3)
Myrcene	123-35-3	1197	A	2.1 (3)	0.4 (1)	5.9 (4)	-	-	-	-	15.2 (4)	-	6.4 (4)	-	-
α-Terpinene	99-86-5	1210	C	9.0 (4)	3.2 (3)	-	-	71.3 (4)	3.8 (4)	48.5 (4)	15.5(4)	18.8 (4)	11.7 4)	30.0 (3)	21.3 (3)
Limonene	138-86-3	1226	C	5.6 (4)	3.2 (3)	-	-	-	-	6.5 (4)	16.6 (4)	-	-	-	-
Eucalyptol	470-82-6	1240	A	-	-	-	-	-	-	-	-	-	-	-	-
γ-Terpinene	99-85-4	1267	A	-	-	-	-	-	-	12.1 (4)	8.8 (4)	-	-	-	-
(E)-Ocimene	3779-61-1	1265	A	0.7 (2)	0.2 (1)	-	-	3.5 (4)	6.6 (4)	-	-	20.3 (4)	6.4 (4)	6.0 (3)	3.2 (2)
p-Cymenene	1195-32-0	1449	A	-	-	1.3 (3)	-	-	-	1.7 (4)	0.8 (4)	-	-	-	-

(Z)-Linalool oxide (furanoid)	34995-77-2	1475	A	0.4 (1)	0.1 (1)	-	1.4 (3)	-	-	-	-	-	-	-	-
Camphor	76-22-2	1541	A	-	-	-	-	-	-	-	-	-	-	-	-
Linalool	78-70-6	1548	C	1.2 (4)	1.3 (3)	1.0 (4)	10.3 (4)	0.1 (3)	0.3 (4)	0.2 (4)	0.6 (4)	0.4 (4)*	0.4 (3)*	0.4 (2)	0.2 (1)
1-methoxy-4-methyl-2-propan-2-ylbenzene	31574-44-4	1587	B	-	-	-	-	1.1 (4)	1.9 (4)	2.3 (4)*	0.6 (4)*	0.7 (4)	tr (3)	0.9 (3)*	0.5 (2)*
Thymol methyl ether	1076-56-8	1601	A	0.9 (4)	0.4 (3)	10.0 (4)	2.7 (4)	4.2 (4)	6.2 (4)	16.1 (4)*	2.9 (4)*	-	-	7.1 (3)*	3.9 (2)*
4-Terpineol	562-74-3	1619	A	-	-	-	-	-	-	-	-	-	-	-	-
Menthol	89-78-1	1642	A	-	-	-	-	-	-	-	-	-	-	-	-
Verbenol	473-67-6	1683	B	-	-	-	-	-	-	-	0.4 (4)	-	-	-	-
α-Terpineol	98-55-5	1703	C	-	-	0.6 (2)	0.9 (1)	0.1 (3)	0.2 (4)	0.2 (4)	0.7 (4)	1.4 (4)*	0.7 (4)*	-	-
Geranial	141-27-5	1685	A	-	-	-	-	-	-	-	-	0.3 (4)	0.4 (4)	-	-
Citronellol	106-22-9	1777	C	-	-	-	-	-	-	-	-	0.4 (4)	0.4 (4)	-	-
Nerol	106-25-2	1815	A	-	-	-	-	-	-	-	-	0.9 (4)	0.5 (4)	-	-
(E,E)-2,6-Dimethylocta-3,5,7-triene-2-ol		1819	A	-	-	-	-	-	-	-	-	0.1 (4)	0.1 (3)	-	-
Geraniol	106-25-2	1850	C	-	-	2.5 (4)	9.6 (4)	-	-	-	-	-	-	-	-
Thymol	89-83-8	2176	C	-	-	-	-	-	0.1 (4)	tr (4)	tr (3)	0.1 (4)	0.1 (3)	-	-
Carvacrol	499-75-2	2206	A	-	-	-	-	tr (1)	0.15 (4)	tr (4)	0.1 (4)	0.3 (4)	0.1 (4)	-	-
Sesquiterpenes															
α-Cubebene	17699-14-8	1473	A	-	-	-	-	-	-	-	-	-	-	-	-
α-Copaene	3856-25-5	1508	A	-	-	-	-	-	-	0.3 (3)	0.2 (3)	-	-	1.6 (3)	-
Caryophyllene	87-44-5	1617	C	-	-	5.0 (4)	-	0.1 (1)	1.2 (4)	0.3 (2)*	0.5 (2)*	1.5 (4)	1.3 (4)	2.1 (3)	-
(E)-β-Farnesene	18794-84-8	1664	A	1.0 (4)	-	-	-	-	-	-	-	-	-	-	-
Humulene	6753-98-6	1690	A	-	-	-	-	-	-	-	-	-	tr (2)	-	-
Verbenone	80-57-9	1720	A	-	-	-	-	-	-	-	0.3 (4)	0.6 (4)	0.3 (4)	-	-
Germacrene D	23986-74-5	1726	A	-	-	-	-	-	-	0.3 (4)	0.2 (4)	-	-	-	-
α-Farnesene	502-61-4	1760	B	-	-	-	-	-	-	-	-	tr (3)	tr (1)	-	-
Caryophyllene epoxide	1139-30-6	2011	C	-	-	-	-	-	-	-	-	-	-	-	-
Ledol	577-27-5	2099	B	-	-	-	-	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,193,119,105,209,91,93,161,43,92,194		1508		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,161,105,91,119,120,81,41,133,93,79		1553		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,93,119,41,69,43,55,91,71,67,79,107		1579		1.5 (4)	0.4 (3)	-	-	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,121,93,107,41,105,91,119,81,94,72		1689		-	-	-	-	-	-	-	-	-	-	-	-
4. SULFUR-CONTAINING COMPOUNDS															
Dimethyl trisulfide	538-86-6	1402	C	-	-	-	-	-	-	-	-	-	-	-	-
5. MISCELLANEOUS															
Nitrogen-containing compounds															
Formanilide	103-70-8	2171	C	-	-	-	-	-	-	-	-	-	-	-	-
Methyl anthranilate	134-20-3	2242	C	-	-	-	-	-	-	-	-	-	-	-	-
Indole	120-72-9	2455	A	-	0.1 (1)	-	-	-	-	tr (2)	0.1 (4)	tr (4)	0.3 (4)	-	-
Unknowns															
m/z: 150*,69,41,81,53,79,107,39,135,82,67		1311		14.0 (2)*	5.1 (2)*	4.1 (4)*	-	-	-	8.8 (4)*	16.7 (4)*	2.6 (4)	0.6 (4)	-	-
m/z: 57,85,41,69,68,32,67,39,53,81		1421		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 73,103,85,69,43,55,57,41,56,74		1453		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 43,59,71,93,55,111,81,41,69,79		1480		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 170*,70,55,83,101,95,96,43,41,39,71		1477		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 57,85,86,93,43,41,55,69,56,80		1569		-	-	-	-	-	-	-	-	-	-	-	-

m/z: 43,57,99,71,149,91,39,41,164,77		1587		-	-	5.9 (4)	1.4 (2)	-	-	-	-	0.7 (4)*	0.1 (3)*	-	-
m/z: 82,81,43,39,54,53,83,41,55,109		1707		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 55,43,83,41,69,57,70,97,81,56		1726		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 79,91,94,59,105,93,77,107,135,150		1680		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 57,97,41,85,81,43,39,53,55,96		1785		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 43,71,41,99,85,59,53,45,81,69		1792		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 57,85,43,35,39,41,67,53,31,58		1989		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 112*,97,35,36,41,112,67,43,39,57,83		2082		-	-	-	-	-	-	-	-	-	-	-	-

Clade				V											
				L. laureolum		*L. microcephalum*		*L. salicifolium*		*L. spissifolium subsp. spissifoium*		*L. uliginosum subsp. uliginosum*		*L. xanthoconus*	
Pollination				Insect		Insect		Wind		Insect		Insect		Insect	
Compounds	CAS #	KRI	Criteria	F	M	F	M	F	M	F	M	F	M	F	M
Number of samples				4	4	3	3	4	4	4	4	4	4	2	2
Average number of compounds				11 ± 0.9	11 ± 0.9	8 ± 0.6	9 ± 1.8	16 ± 0.8	9 ± 2.1	19 ± 1.8	24 ± 1.7	17 ± 2.4	20 ± 2.7	24 ± 2.5	24 ± 0.5
Median of scent emission per flower (ng/h)				1.07	0.07	0.15	0.03	0.63	0.04	2.26	1.05	0.32	0.06	5.59	0.52
Median of scent emission per inflorescence (ng/h)				56.8	34.1	5.4	8.5	32.7	4.8	142.3	119.8	20.6	13.8	184.4	63.2
1. ALIPHATIC COMPOUNDS															
Alcohols															
n-Pentan-1-ol	71-41-0	1254	A	1.7 (2)	6.8 (4)	-	19.6 (3)	-	-	-	-	-	-	-	-
Heptan-2-ol	72237-36-6	1321	A	-	-	-	-	-	-	-	-	-	-	65.7 (2)	40.9 (2)
Hexan-1-ol	928-96-1	1352	C	3.2 (4)	5.0 (4)	-	17.0 (3)	-	-	-	-	-	-	0.4 (2)	0.3 (2)
(Z)-Hex-3-en-1-ol	3658-80-8	1381	A	-	-	-	-	2.0 (4)	7.2 (3)	4.1 (4)*	5.4 (4)*	-	-	0.2 (2)*	0.2 (2)*
(E)-Hex-2-en-1-ol	928-95-0	1423	A	-	-	-	-	-	-	2.0 (4)	14.9 (4)	0.4 (4)*	0.3 (3)*	0.2 (2)	0.2 (2)
Oct-1-en-3-ol	3391-86-4	1462	C	1.7 (4)	1.0 (4)	-	-	-	-	-	-	-	-	-	-
n-Heptan-1-ol	111-70-6	1453	A	0.5 (2)	1.8 (4)	-	-	-	-	-	-	-	-	-	-
Aldehydes															
Pentanal	110-62-3	1076	A	-	-	-	35.0 (2)	-	-	-	-	-	-	-	-
(Z)-Hex-3-enal	6789-80-6	1193	A	-	-	-	-	-	-	-	-	-	-	-	-
Heptanal	111-71-7	1221	A	28.45 (4)*	41.5 (4)*	-	16.8 (2)	-	-	-	-	-	-	-	-
(E)-Hex-2-enal	6728-26-3	1232	A	-	-	-	-	-	-	4.5 (4)	2.4 (4)	-	-	-	-
Esters															
Butyl acetate	123-86-4	1138	C	-	-	-	-	-	-	-	-	-	-	-	-
Methyl hexanoate	106-70-7	1221	A	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl caproate	123-66-0	1250	A	-	-	-	-	6.4 (4)	-	-	-	-	-	-	-
2-Heptyl acetate	5921-82-4	1288	A	-	-	-	-	-	-	-	-	-	-	1.5 (1)	1.2 (2)
Methyl (Z)-3-hexenoate	2396-78-3	1282	A	-	-	-	-	-	-	-	-	-	-	5.2 (2)	13.4 (2)
Hexyl acetate	124-92-7	1297	C	-	-	-	-	-	-	1.7 (3)*	0.7 (2)*	-	-	-	-
Methyl 2-hexenoate	13894-63-8	1303	A	-	-	-	-	13.3 (4)	0.4 (2)	4.2 (4)	8.5 (4)	-	-	0.8 (2)	-

Isoamyl isovalerate	659-70-1	1305	B	-	-	-	-	-	-	-	-	-	-	-	-
Hex-4-en-1-yl acetate	10094-40-3	1325	B	-	-	-	-	3.4 (4)	3.6 (3)	45.5 (4)	-	-	10.5 (3)	-	-
(E)-Hex-2-en-1-yl acetate	111-27-3	1350	A	-	-	-	-	-	-	-	-	-	-	-	-
3-Cyclohexen-1-ol, acetate	10437-78-2	1449	B	-	-	-	-	-	-	-	-	-	-	0.1 (2)	0.2 (2)
Methyl 2-hydroxy-3-methylpentanoate	41654-19-7	1531	B	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl formate	104-57-4	1709	A	-	-	-	-	-	-	-	-	-	-	-	-
3-hydroxy-2,4,4-trimethylpentyl 2-methyl-propanoate	74367-34-3	1884	B	-	-	2.2 (3)	1.5 (3)	-	-	-	-	0.2 (4)*	0.2 (4)*	-	-
Unidentified aliphatic ester *m/z*: 158*,57,85,98,103,129,41,70,56,55,74		1425		-	-	-	-	-	-	-	-	-	-	0.1 (1)	0.1 (2)
Unidentified aliphatic ester *m/z*: 85,57,41,43,98,56,119,60,102,55		1446		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 55,83,101,95,70,96,43,41,39,71		1488		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 70,55,83,101,95,96,43,41,39,71		1536		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 57,91,41,71,85,43,108,79,32,39		1905		-	-	-	-	-	-	-	-	-	-	-	-
Ketones															
Heptan-2-one	110-43-0	1210	B	-	-	-	-	-	-	9.8 (4)	18.2 (4)	-	-	6.7 (2)	-
2-Octanone	111-13-7	1300	A	-	-	-	-	-	-	-	-	-	-	-	-
6,10-Dimethyl-5,9-undecadien-2-one	686-67-8	1870	B	-	-	-	-	-	-	-	-	-	-	-	-
cis-Jasmone	488-10-8	1967	A	-	-	-	-	-	-	-	0.1 (3)	-	0.5 (3)	-	-
2-Ethylcyclohexanone	4423-94-3	2114		-	-	-	-	-	-	-	-	0.2 (3)	0.3 (4)	-	-
2. BENZENOIDS AND PHENYLPROPANOIDS															
Benzyl methyl ether	538-86-3	1412	A	-	-	-	-	4.9 (4)*	-	-	-	-	-	-	-
Benzaldehyde	100-52-7	1537	C	-	-	-	-	8.6 (4)*	-	4.5 (4)*	9.2 (4)*	-	-	-	-
Methylbenzoate	93-58-3	1634	C	0.2 (1)	0.2 (2)	2.7 (2)	0.7 (2)	14.6 (4)*	7.3 (3)*	0.6 (4)*	0.3 (4)*	tr (1)	-	-	-
Estragole	140-67-0	1688	A	-	-	-	-	-	-	-	-	-	0.1 (3)	-	-
1,2-Dimethoxybenzene (veratrole)	91-16-7	1676	A	-	-	-	-	2.6 (4)	2.2 (3)	-	-	-	-	-	-
Benzyl acetate	140-11-4	1678	C	-	-	-	-	-	-	-	-	-	-	-	-
Methyl phenylacetate	101-41-7	1770	A	-	-	-	-	-	-	1.4 (4)	4.3 (4)	-	-	-	-
Methyl salicylate	119-36-8	1792	C	-	-	-	-	-	-	-	-	tr (2)	-	-	-
Phenylethyl acetate	103-45-7	1839	A	-	-	-	-	-	-	-	-	tr (1)	-	-	-
p-Cymen-8-ol	1197-01-9	1853	A	-	-	-	-	-	-	-	-	3.0 (4)*	1.8 (4)*	0.5 (2)*	0.5 (2)*
Guaiacol	90-05-1	1870	A	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol	100-51-6	1882	C	-	-	-	2.8 (3)	-	-	2.2 (3)*	3.3 (4)*	-	-	-	-
Phenylethyl alcohol	60-12-8	1920	A	-	-	-	-	0.2 (3)	0.2 (3)	-	-	0.1 (4)*	tr (1)*	tr (2)	-
Phenol	108-95-2	2026	A	-	-	-	1.2 (3)	-	-	1.4 (2)*	10.8 (4)*	-	-	-	-
Methyleugenol	93-15-2	2026	A	-	-	-	-	-	-	-	-	tr (2)	0.2 (3)	-	-
4-Methoxybenzaldehyde	123-11-5	2037	B	-	-	-	-	-	-	-	1.0 (4)	-	-	-	-
p-Cresol	106-44-5	2098	A	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl tiglate	37526-88-8	2121	B	-	-	-	-	-	-	-	-	-	-	-	-
1,3,5-Trimethyoxybenzene	621-23-8	2151	B	-	-	-	-	-	-	0.2 (4)	0.6 (4)	-	-	-	-
3. TERPENOIDS															
Monoterpenes															
α-Pinene	80-56-8	1109	C	-	-	-	-	-	-	-	-	44.9 (4)	29.1 (4)	5.8 (2)	9.9 (2)
Sabinene	3387-41-5	1157	A	27.2 (4)	17.2 (4)	-	-	-	-	-	-	21.9 (4)	16.9 (4)	4.7 (2)	9.5 (2)
Myrcene	123-35-3	1197	A	16.9 (4)	3.1 (2)	-	-	-	-	-	-	-	0.8 (3)	0.1 (1)	-
α-Terpinene	99-86-5	1210	C	-	-	-	-	-	-	-	-	8.0 (4)	10.2 (3)	-	15.1 (2)
Limonene	138-86-3	1226	C	-	-	-	-	-	-	-	-	3.1 (4)*	1.4 (3)*	-	-
Eucalyptol	470-82-6	1240	A	-	-	-	-	-	-	-	-	-	-	3.1 (2)	4.2 (2)
γ-Terpinene	99-85-4	1267	A	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Ocimene	3779-61-1	1265	A	2.9 (4)	12.8 (4)	1.8 (1)	-	4.4 (2)*	29.1 (3)*	5.7 (4)	3.1 (3)	6.8 (4)	18.9 (4)	2.8 (2)	1.0 (2)

p-Cymenene	1195-32-0	1449	A	-	-	-	-	-	-	-	-	1.5 (3)	0.8 (4)	1.4 (2)	2.4 (2)
(Z)-Linalool oxide (furanoid)	34995-77-2	1475	A	-	-	-	-	-	-	-	-	1.4 (3)	0.4 (2)	-	-
Camphor	76-22-2	1541	A	-	-	-	-	-	-	-	-	-	-	-	-
Linalool	78-70-6	1548	C	2.8 (4)	1.5 (3)	-	-	0.7 (4)	2.1 (4)	5.2 (4)	8.2 (4)	0.3 (3)*	0.8 (4)*	-	-
1-methoxy-4-methyl-2-propan-2-ylbenzene	31574-44-4	1587	B	-	-	-	-	-	-	0.2 (3)	0.3 (2)	0.4 (3)	1.0 (4)	-	-
Thymol methyl ether	1076-56-8	1601	A	3.5 (4)	0.8 (2)	3.7 (3)	3.4 (2)	-	-	1.1 (4)	1.8 (4)	4.7 (4)	4.3 (4)	tr (1)	tr (2)
4-Terpineol	562-74-3	1619	A	-	-	-	-	-	-	-	-	-	-	0.1 (2)	0.1 (2)
Menthol	89-78-1	1642	A	-	-	-	-	-	-	-	0.2 (3)	-	-	-	-
Verbenol	473-67-6	1683	B	-	-	-	-	-	-	-	-	-	-	-	-
α-Terpineol	98-55-5	1703	C	-	-	-	-	-	-	-	-	0.5 (2)*	0.3 (3)*	tr (2)*	tr (2)*
Geranial	141-27-5	1685	A	-	-	-	-	-	-	-	-	-	-	-	-
Citronellol	106-22-9	1777	C	-	-	-	-	-	-	-	-	-	-	-	-
Nerol	106-25-2	1815	A	-	-	-	-	-	-	-	-	-	-	-	-
(E,E)-2,6-Dimethylocta-3,5,7-triene-2-ol		1819	A	-	-	-	-	0.2 (3)	-	0.1 (2)	0.1 (1)	0.1 (1)	0.1 (2)	-	-
Geraniol	106-25-2	1850	C	-	-	-	-	-	-	0.1 (1)	0.9 (3)	-	-	-	-
Thymol	89-83-8	2176	C	-	-	-	-	-	-	-	-	tr (2)	tr (3)	tr (1)	tr (2)
Carvacrol	499-75-2	2206	A	-	-	-	-	-	-	-	-	tr (3)	tr (1)	tr (1)	tr (2)
Sesquiterpenes															
α-Cubebene	17699-14-8	1473	A	-	-	-	-	-	-	-	-	-	-	-	-
α-Copaene	3856-25-5	1508	A	-	-	-	-	-	-	-	-	-	-	-	-
Caryophyllene	87-44-5	1617	C	3.1 (4)	4.7 (4)	19.5 (3)	1.2 (2)	24.6 (4)	44.5 (4)	5.4 (4)	3.9 (4)	1.3 (4)	0.6 (4)	0.2 (2)	0.1 (2)
(E)-β-Farnesene	18794-84-8	1664	A	-	-	-	-	-	-	-	-	-	-	-	-
Humulene	6753-98-6	1690	A	-	-	1.9 (3)	-	0.6 (4)	0.6 (3)	0.1 (2)	0.1 (1)	-	-	0.1 (1)	0.1 (1)
Verbenone	80-57-9	1720	A	-	-	-	-	-	-	-	-	-	-	-	-
Germacrene D	23986-74-5	1726	A	-	-	-	-	-	-	-	-	-	-	-	-
α-Farnesene	502-61-4	1760	B	-	-	1.1 (3)	-	0.1 (2)	0.2 (2)	-	-	0.1 (2)*	0.2 (4)*	0.1 (2)	0.1 (2)
Caryophyllene epoxide	1139-30-6	2011	C	-	-	-	-	-	-	-	-	-	-	-	-
Ledol	577-27-5	2099	B	1.3 (2)	-	-	-	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,193,119,105,209,91,93,161,43,92,194		1508		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,161,105,91,119,120,81,41,133,93,79		1553		-	-	-	-	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,93,119,41,69,43,55,91,71,67,79,107		1579		-	-	4.1 (3)	0.3 (2)	-	-	-	-	-	-	-	-
Unidentified sesquiterpene *m/z*: 204*,121,93,107,41,105,91,119,81,94,72		1689		-	-	-	-	-	-	-	-	-	-	-	-
4. SULFUR-CONTAINING COMPOUNDS															
Dimethyl trisulfide	538-86-6	1402	C	-	-	-	-	1.0 (4)	-	-	-	-	-	-	-
5. MISCELLANEOUS															
Nitrogen-containing compounds															
Formanilide	103-70-8	2171	C	-	-	-	-	-	-	-	0.3 (3)	-	-	-	-
Methyl anthranilate	134-20-3	2242	C	-	-	-	-	-	-	-	0.1 (2)	-	-	-	-
Indole	120-72-9	2455	A	-	-	-	-	-	-	tr (1)*	0.5 (2)*	-	-	-	-
Unknowns															
m/z: 150*,69,41,81,53,79,107,39,135,82,67		1311		6.4 (1)	3.7 (3)	63.2 (3)	0.6 (1)	6.7 (3)	1.4 (2)	-	-	1.0 (2)*	0.4 (1)*	0.2 (2)	0.4 (2)
m/z: 57,85,41,69,68,32,67,39,53,81		1421		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 73,103,85,69,43,55,57,41,56,74		1453		-	-	-	-	5.8 (4)*	1.2 (1)*	-	-	-	-	-	-
m/z: 43,59,71,93,55,111,81,41,69,79		1480		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 170*,70,55,83,101,95,96,43,41,39,71		1477		-	-	-	-	-	-	-	-	-	-	-	-

m/z: 57,85,86,93,43,41,55,69,56,80		1569		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 43,57,99,71,149,91,39,41,164,77		1587		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 82,81,43,39,54,53,83,41,55,109		1707		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 55,43,83,41,69,57,70,97,81,56		1726		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 79,91,94,59,105,93,77,107,135,150		1680		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 57,97,41,85,81,43,39,53,55,96		1785		-	-	-	-	-	-	-	-	-	-	-	-
m/z: 43,71,41,99,85,59,53,45,81,69		1792		-	-	-	-	-	-	0.1 (3)	0.2 (4)	-	-	-	-
m/z: 57,85,43,35,39,41,67,53,31,58		1989		-	-	-	-	-	-	-	0.3 (3)	-	-	-	-
m/z: 112*,97,35,36,41,112,67,43,39,57,83		2082		-	-	-	-	-	-	tr (1)	0.5 (4)	-	-	-	-

Clade				VI				VII		VIII			
				L. sessile		*L. tinctum*		*L. linifolium*		*L. pubescens*		*L. salignum*	
Pollination				Insect		Insect		Insect		Insect		Insect	
Compounds	CAS #	KRI	Criteria	F	M	F	M	F	M	F	M	F	M
Number of samples				3	3	4	4	4	4	2	2	4	4
Average number of compounds				28 ± 2.6	29 ± 5.2	35 ± 0.5	29 ± 0.5	15 ± 2.1	12 ± 1.9	13 ± 0.5	14 ± 1.0	20 ± 0.6	15 ± 2.6
Median of scent emission per flower (ng/h)				15.13	3.37	13.34	0.64	2.87	1.41	2.93	0.42	4.41	0.27
Median of scent emission per inflorescence (ng/h)				317.7	1290.5	693.7	140.8	68.8	148.2	73.3	41.9	141.3	42.4
1. ALIPHATIC COMPOUNDS													
Alcohols													
n-Pentan-1-ol	71-41-0	1254	A	-	-	0.1 (4)*	0.3 (4)*	-	-	-	-	-	-
Heptan-2-ol	72237-36-6	1321	A	-	-	-	-	-	-	-	-	-	-
Hexan-1-ol	928-96-1	1352	C	2.6 (3)*	1.0 (3)*	0.2 (4)	0.6 (4)	-	-	-	-	-	-
(Z)-Hex-3-en-1-ol	3658-80-8	1381	A	3.8 (3)*	1.1 (2)*	3.5 (3)*	0.9 (2)*	-	-	-	-	-	-
(E)-Hex-2-en-1-ol	928-95-0	1423	A	-	-	-	-	-	-	-	4.7 (2)*	0.4 (4)	1.6 (4)
Oct-1-en-3-ol	3391-86-4	1462	C	-	-	-	-	-	-	-	-	0.4 (4)	0.3 (4)
n-Heptan-1-ol	111-70-6	1453	A	-	-	-	-	-	-	-	-	-	-
Aldehydes													
Pentanal	110-62-3	1076	A	-	-	-	-	-	-	-	-	-	-
(Z)-Hex-3-enal	6789-80-6	1193	A	-	-	-	-	-	-	21.7 (2)	-	-	-
Heptanal	111-71-7	1221	A	-	-	-	-	-	-	-	-	-	-
(E)-Hex-2-enal	6728-26-3	1232	A	-	-	-	-	-	-	-	50.7 (2)*	-	3.5 (4)
Esters													
Butyl acetate	123-86-4	1138	C	0.3 (3)	tr (2)	-	-	-	-	5.5 (2)*	6.7 (2)*	-	-
Methyl hexanoate	106-70-7	1221	A	-	-	1.0 (3)	4.0 (4)	-	-	-	-	-	-
Ethyl caproate	123-66-0	1250	A	-	-	-	-	-	-	-	-	-	-
2-Heptyl acetate	5921-82-4	1288	A	-	-	-	-	-	-	-	-	-	-
Methyl (Z)-3-hexenoate	2396-78-3	1282	A	-	-	0.5 (4)	2.6 (4)	-	-	-	-	-	-
Hexyl acetate	124-92-7	1297	C	4.1 (3)	2.3 (3)	1.4 (4)	1.7 (4)	-	-	-	-	-	-
Methyl 2-hexenoate	13894-63-8	1303	A	-	-	-	-	4.6 (3)	-	-	0.8 (2)	21.3 (4)	14.1 (4)
Isoamyl isovalerate	659-70-1	1305	B	-	-	-	-	22.8 (4)	-	-	-	-	-

Hex-4-en-1-yl acetate	10094-40-3	1325	B	20.3 (2)*	29.0 (3)*	63.5 (4)*	48.7 (4)*	-	-	-	-	-	-
(E)-Hex-2-en-1-yl acetate	111-27-3	1350	A	-	-	-	-	-	-	-	-	-	-
3-Cyclohexen-1-ol, acetate	10437-78-2	1449	B	-	-	-	-	-	-	-	-	-	-
Methyl 2-hydroxy-3-methylpentanoate	41654-19-7	1531	B	-	-	-	-	-	-	-	-	-	-
Benzyl formate	104-57-4	1709	A	tr (2)	tr (2)	-	-	-	-	-	-	-	-
3-hydroxy-2,4,4-trimethylpentyl 2-methyl-propanoate	74367-34-3	1884	B	-	-	tr (2)*	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 158*,57,85,98,103,129,41,70,56,55,74		1425		-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 85,57,41,43,98,56,119,60,102,55		1446		-	-	-	-	-	-	-	-	-	-
Unidentified aliphatic ester *m/z*: 55,83,101,95,70,96,43,41,39,71		1488		-	-	-	-	-	-	0.4 (2)	-	-	-
Unidentified aliphatic ester *m/z*: 70,55,83,101,95,96,43,41,39,71		1536		-	-	-	-	0.5 (4)	0.1 (3)	-	-	-	-
Unidentified aliphatic ester *m/z*: 57,91,41,71,85,43,108,79,32,39		1905		-	-	-	-	0.2 (3)	0.1 (3)	-	-	-	-
Ketones													
Heptan-2-one	110-43-0	1210	B	-	-	-	-	-	-	-	-	-	-
2-Octanone	111-13-7	1300	A	-	-	-	-	-	-	-	-	-	-
6,10-Dimethyl-5,9-undecadien-2-one	686-67-8	1870	B	tr (3)	-	tr (4)	tr (4)	-	-	-	-	0.2 (4)	-
cis-Jasmone	488-10-8	1967	A	-	-	-	-	-	-	-	-	-	-
2-Ethylcyclohexanone	4423-94-3	2114		tr (2)	tr (2)	-	-	-	-	-	-	-	-
2. BENZENOIDS AND PHENYLPROPANOIDS													
Benzyl methyl ether	538-86-3	1412	A	-	-	-	-	-	-	-	-	-	-
Benzaldehyde	100-52-7	1537	C	36.5 (3)*	24.3 (3)*	-	-	-	-	-	-	-	-
Methylbenzoate	93-58-3	1634	C	9.3 (3)	8.4 (3)	0.2 (4)*	3.6 (4)*	1.0 (3)	0.1 (2)	-	-	14.8 (4)	1.8 (3)
Estragole	140-67-0	1688	A	-	-	-	-	-	-	-	-	-	-
1,2-Dimethoxybenzene (veratrole)	91-16-7	1676	A	-	-	-	-	-	-	-	-	-	-
Benzyl acetate	140-11-4	1678	C	tr (3)	tr (2)	-	-	-	-	-	-	-	-
Methyl phenylacetate	101-41-7	1770	A	-	-	tr (4)	0.1 (3)	-	-	-	-	0.1 (1)	0.3 (3)
Methyl salicylate	119-36-8	1792	C	-	-	-	-	0.1 (2)	-	-	-	-	-
Phenylethyl acetate	103-45-7	1839	A	-	-	-	-	-	-	-	-	-	-
p-Cymen-8-ol	1197-01-9	1853	A	tr (2)*	tr (3)*	tr (4)*	0.1 (3)*	-	-	0.2 (2)	tr (1)	-	-
Guaiacol	90-05-1	1870	A	-	-	-	-	-	tr (3)	-	-	-	-
Benzyl alcohol	100-51-6	1882	C	8.0 (3)*	10.9 (3)*	-	-	1.4 (3)*	2.3 (4)*	-	-	-	-
Phenylethyl alcohol	60-12-8	1920	A	tr (2)	tr (2)	-	-	0.6 (4)	0.2 (4)	-	-	-	-
Phenol	108-95-2	2026	A	-	-	-	-	30.1 (4)*	75.4 (4)*	36.8 (2)*	17.8 (1)*	-	-
Methyleugenol	93-15-2	2026	A	-	-	-	-	-	-	-	-	-	-
4-Methoxybenzaldehyde	123-11-5	2037	B	-	-	-	-	-	-	-	-	-	-
p-Cresol	106-44-5	2098	A	tr (1)	tr (2)	-	-	-	-	-	-	-	-
Benzyl tiglate	37526-88-8	2121	B	-	-	-	-	tr (2)	0.2 (4)	-	-	-	-
1,3,5-Trimethyoxybenzene	621-23-8	2151	B	-	-	-	-	-	-	-	-	-	-
3. TERPENOIDS													
Monoterpenes													
α-Pinene	80-56-8	1109	C	0.4 (1)*	2.5 (3)*	-	-	-	-	-	-	-	-
Sabinene	3387-41-5	1157	A	4.0 (3)	9.5 (3)	1.0 (4)	11.0 (4)	-	-	13.4 (2)	-	-	-
Myrcene	123-35-3	1197	A	0.6 (2)	1.9 (3)	-	-	-	-	-	-	-	-
α-Terpinene	99-86-5	1210	C	-	1.8 (3)	-	-	-	-	4.1 (2)	-	-	-
Limonene	138-86-3	1226	C	0.3 (1)*	0.5 (2)*	-	-	24.2	18.9	10.5	-	-	-

								(2)*	(3)*	(2)*			
Eucalyptol	470-82-6	1240	A	-	-	-	-	-	-	-	-	-	-
γ-Terpinene	99-85-4	1267	A	tr (1)	0.1 (1)	-	-	-	-	-	-	-	-
(E)-Ocimene	3779-61-1	1265	A	4.3 (3)*	2.0 (3)*	1.9 (4)	-	-	-	-	2.9 (1)	38.1 (4)	54.7 (4)
p-Cymenene	1195-32-0	1449	A	0.1 (2)	0.1 (2)	-	-	-	-	-	-	-	-
(Z)-Linalool oxide (furanoid)	34995-77-2	1475	A	tr (1)	tr (2)	1.9 (4)	2.1 (4)	-	-	-	0.1 (1)	-	-
Camphor	76-22-2	1541	A	-	-	tr (4)	tr (3)	-	-	-	-	-	-
Linalool	78-70-6	1548	C	1.0 (3)*	0.5 (2)*	1.1 (4)*	1.6 (4)*	0.3 (2)*	-	0.8 (2)*	0.1 (2)*	0.1 (4)*	11.9 (3)*
1-methoxy-4-methyl-2-propan-2-ylbenzene	31574-44-4	1587	B	-	-	tr (4)*	tr (3)	-	-	0.7 (2)	0.2 (2)	-	-
Thymol methyl ether	1076-56-8	1601	A	0.1 (3)	0.1 (2)	0.4 (4)*	0.3 (4)*	0.2 (2)*	-	4.4 (2)	1.3 (2)	0.2 (3)	0.3 (2)
4-Terpineol	562-74-3	1619	A	-	-	-	-	-	-	-	-	-	-
Menthol	89-78-1	1642	A	-	-	-	-	-	-	-	-	-	-
Verbenol	473-67-6	1683	B	-	-	-	-	-	-	-	-	-	-
α-Terpineol	98-55-5	1703	C	0.8 (3)*	2.1 (3)*	3.3 (4)*	1.5 (4)*	-	-	-	-	-	-
Geranial	141-27-5	1685	A	tr (3)	tr (2)	-	-	-	-	-	-	-	-
Citronellol	106-22-9	1777	C	-	-	-	-	-	-	-	-	-	-
Nerol	106-25-2	1815	A	-	-	-	-	-	-	-	-	-	-
(E,E)-2,6-Dimethylocta-3,5,7-triene-2-ol		1819	A	0.1 (2)	tr (2)	-	-	-	-	-	-	0.6 (4)	1.0 (3)
Geraniol	106-25-2	1850	C	-	tr (2)	-	-	-	-	-	-	0.3 (4)	0.1 (2)
Thymol	89-83-8	2176	C	-	tr (2)	tr (4)	0.1 (4)	-	-	-	-	-	-
Carvacrol	499-75-2	2206	A	-	-	-	-	-	-	-	-	-	-
Sesquiterpenes													
α-Cubebene	17699-14-8	1473	A	-	-	0.1 (3)	0.3 (2)	-	-	-	-	-	-
α-Copaene	3856-25-5	1508	A	-	-	1.2 (4)	1.5 (4)	-	-	-	-	-	-
Caryophyllene	87-44-5	1617	C	1.8 (3)	1.3 (3)	1.5 (4)	2.8 (4)	8.4 (4)	0.4 (4)	-	-	15.7 (4)	5.6 (4)
(E)-β-Farnesene	18794-84-8	1664	A	-	-	-	-	-	-	-	-	-	-
Humulene	6753-98-6	1690	A	tr (2)	tr (3)	0.1 (4)	0.1 (4)	0.2 (4)	-	-	-	0.4 (4)	0.1 (2)
Verbenone	80-57-9	1720	A	-	-	-	-	-	-	-	-	-	-
Germacrene D	23986-74-5	1726	A	-	-	0.3 (4)	0.3 (4)	-	-	-	-	0.2 (4)	0.1 (2)
α-Farnesene	502-61-4	1760	B	tr (3)	0.2 (2)	tr (4)	0.1 (4)	0.2 (3)	-	-	-	0.4 (4)	0.4 (1)
Caryophyllene epoxide	1139-30-6	2011	C	-	-	tr (3)	-	-	-	-	-	0.2 (4)	-
Ledol	577-27-5	2099	B	-	-	-	-	-	-	-	-	-	-
Unidentifided sesquiterpene *m/z*: 204*,193,119,105,209,91,93,161,43,92,194		1508		-	-	0.1 (4)	0.2 (3)	-	-	-	-	-	-
Unidentifided sesquiterpene *m/z*: 204*,161,105,91,119,120,81,41,133,93,79		1553		-	-	0.4 (4)	0.2 (4)	-	-	-	-	-	-
Unidentifided sesquiterpene *m/z*: 204*,93,119,41,69,43,55,91,71,67,79,107		1579		-	-	-	-	-	-	-	-	-	-
Unidentifided sesquiterpene *m/z*: 204*,121,93,107,41,105,91,119,81,94,72		1689		-	-	tr (3)	0.1 (2)	-	-	-	-	-	-
4. SULFUR-CONTAINING COMPOUNDS													
Dimethyl trisulfide	538-86-6	1402	C	-	-	-	-	-	-	-	-	-	-
5. MISCELLANEOUS													
Nitrogen-containing compounds													
Formanilide	103-70-8	2171	C	-	-	-	-	-	-	-	-	0.1 (2)	0.3 (2)
Methyl anthranilate	134-20-3	2242	C	-	-	tr (2)	tr (2)	-	-	-	-	-	-
Indole	120-72-9	2455	A	-	-	0.3 (4)	3.7 (4)	-	-	-	tr (2)	1.2 (4)	0.1 (3)
Unknowns													
m/z: 150*,69,41,81,53,79,107,39,135,82,67		1311		1.1 (3)*	0.4 (3)*	15.8 (4)*	11.3 (4)*	4.7 (3)	2.3 (4)	-	-	4.7 (4)	3.2 (3)

m/z: 57,85,41,69,68,32,67,39,53,81	1421	-	-	-	-	0.1 (2)	tr (3)	-	-	-	-
m/z: 73,103,85,69,43,55,57,41,56,74	1453	-	-	-	-	-	-	1.0 (2)	0.2 (2)	-	-
m/z: 43,59,71,93,55,111,81,41,69,79	1480	0.3 (3)	-	0.1 (4)	0.3 (3)	-	-	-	-	-	-
m/z: 170*,70,55,83,101,95,96,43,41,39,71	1477	-	-	-	-	0.5 (4)	0.1 (3)	-	-	-	-
m/z: 57,85,86,93,43,41,55,69,56,80	1569	-	-	0.1 (4)*	-	-	-	-	5.8 (2)	-	-
m/z: 43,57,99,71,149,91,39,41,164,77	1587	-	-	-	-	-	-	-	-	-	-
m/z: 82,81,43,39,54,53,83,41,55,109	1707	-	-	-	-	-	-	-	-	0.2 (4)	0.2 (2)
m/z: 55,43,83,41,69,57,70,97,81,56	1726	-	-	-	-	0.3 (3)	0.1 (2)	-	-	-	-
m/z: 79,91,94,59,105,93,77,107,135,150	1680	-	-	-	-	-	-	-	-	0.2 (3)	0.2 (3)
m/z: 57,97,41,85,81,43,39,53,55,96	1785	-	-	-	-	-	-	-	0.3 (2)*	-	-
m/z: 43,71,41,99,85,59,53,45,81,69	1792	0.1 (2)	tr (2)	tr (3)*	-	-	-	-	-	0.4 (4)	0.4 (3)
m/z: 57,85,43,35,39,41,67,53,31,58	1989	-	-	-	-	-	-	0.5 (1)	8.6 (2)	-	-
m/z: 112*,97,35,36,41,112,67,43,39,57,83	2082	-	-	-	-	-	-	-	-	-	-

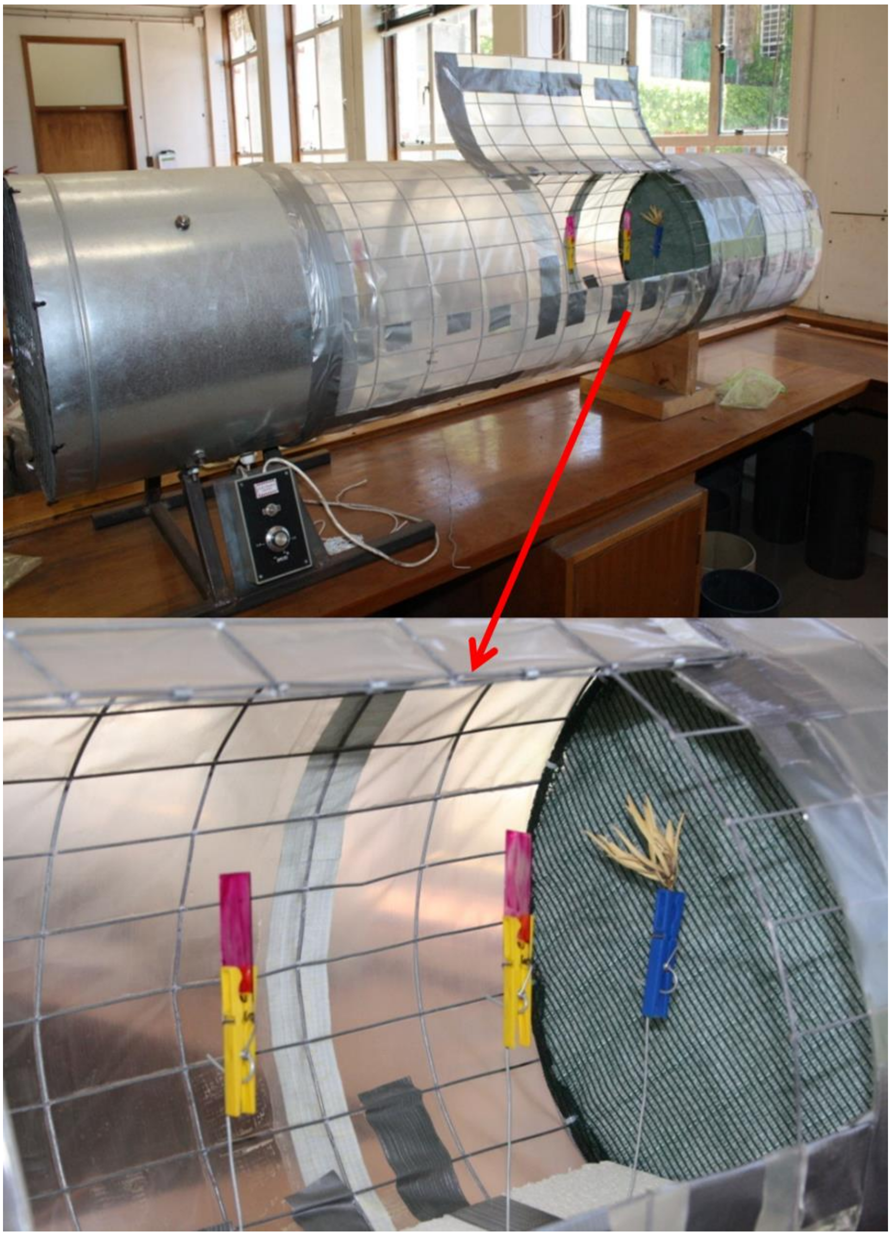

Figure S1. A tubular wind tunnel with laminar flow used to determine pollen motility (diameter: 45cm, and total length: 188cm).

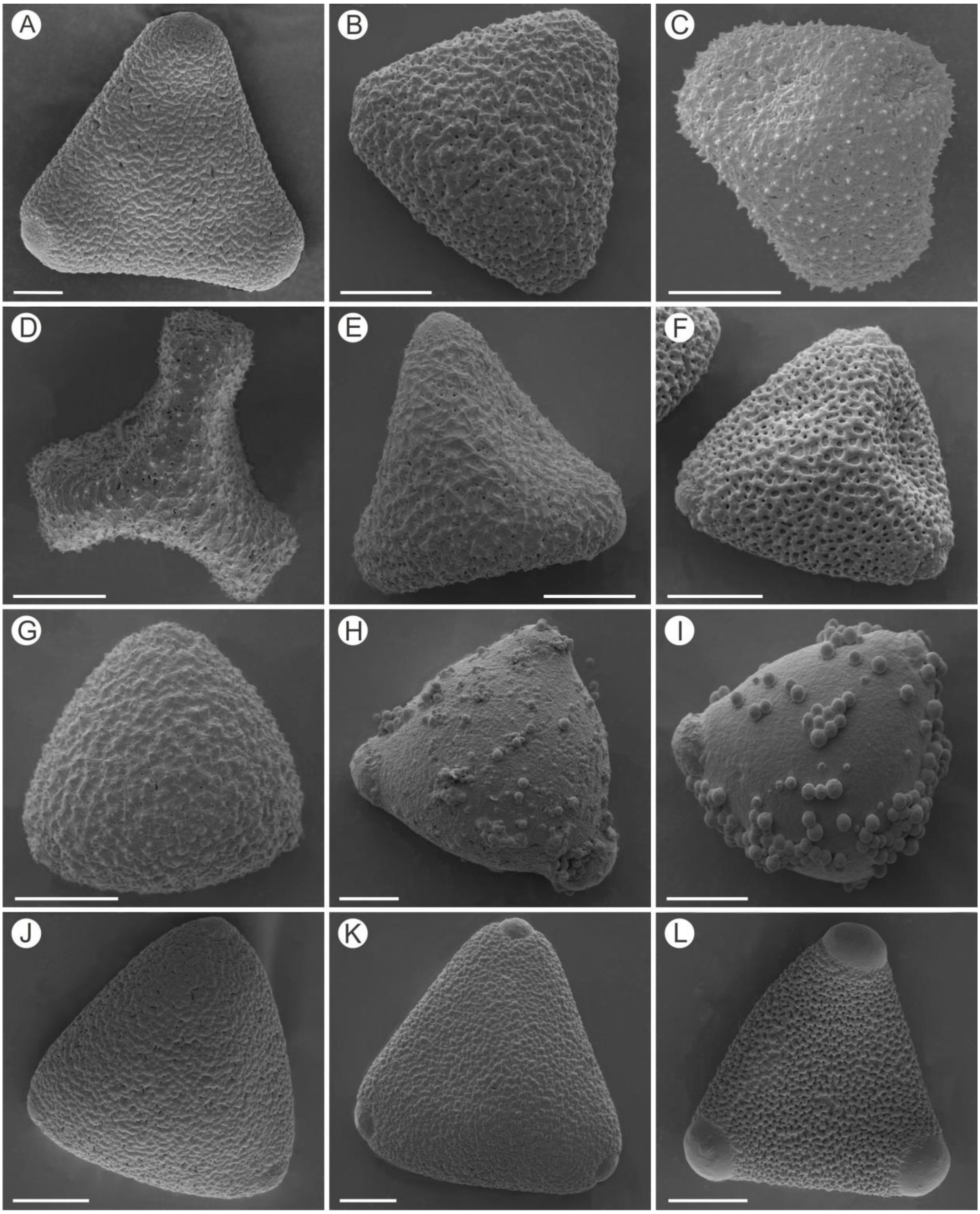

Figure S2. Scanning electron micrograph of pollen grains from insect-pollinated *Leucadendron* species: (A) *L. argenteum*; (B) *L. platyspermum*; (C) *L. laureolum* (D) *L. microcephalum*; (E) *L. spissifolium* subsp. *spissifolium*; (F) *L. uliginosum* subsp. *uliginosum*; (G) *L. xanthoconus*; (H) *L. sessile*; (H) *L. tinctum*; (I) *L. linifolium*; (K) *L. pubescens*; (L) *L. salignum.* Scale bars: 10 µm.

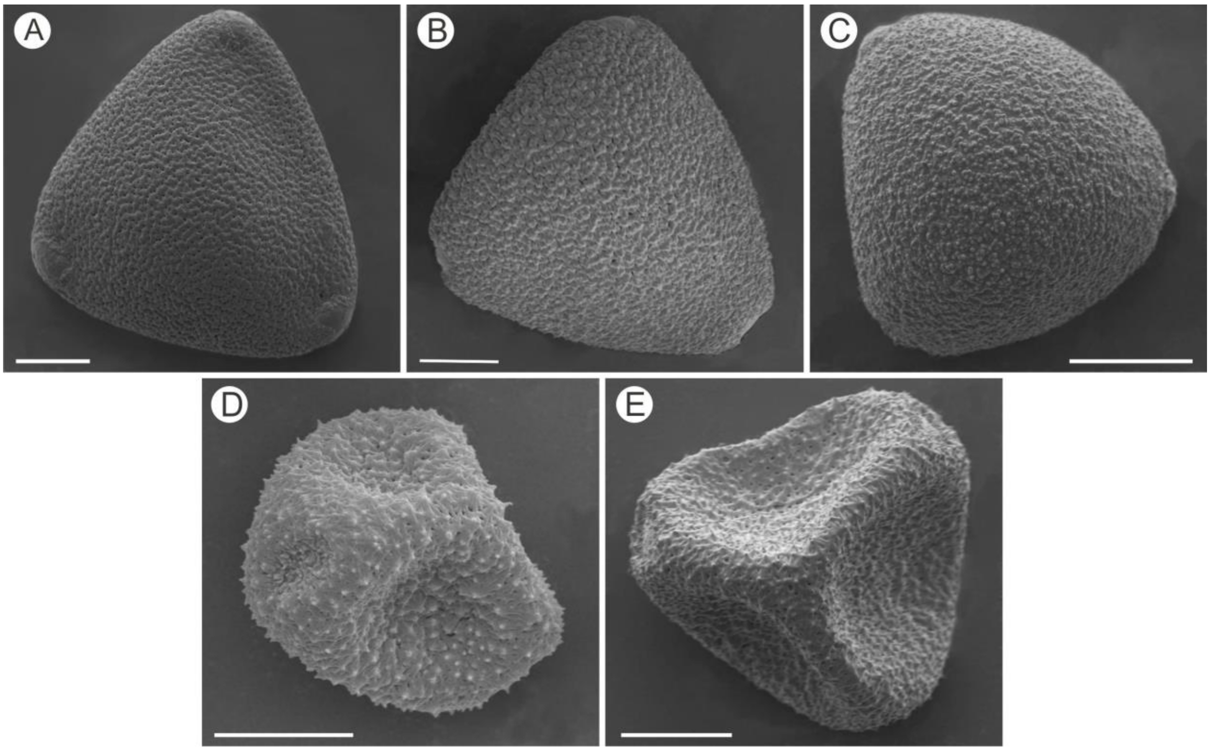

Figure. S3. Scanning electron micrograph of pollen grains from wind-pollinated *Leucadendron* species: (A) *L. dubium*; (B) *L. rubrum*; (C) *L. teretifolium*; (D) *L. coniferum*; (E) *L. salicifolium*. Scale bars: 10 μm.

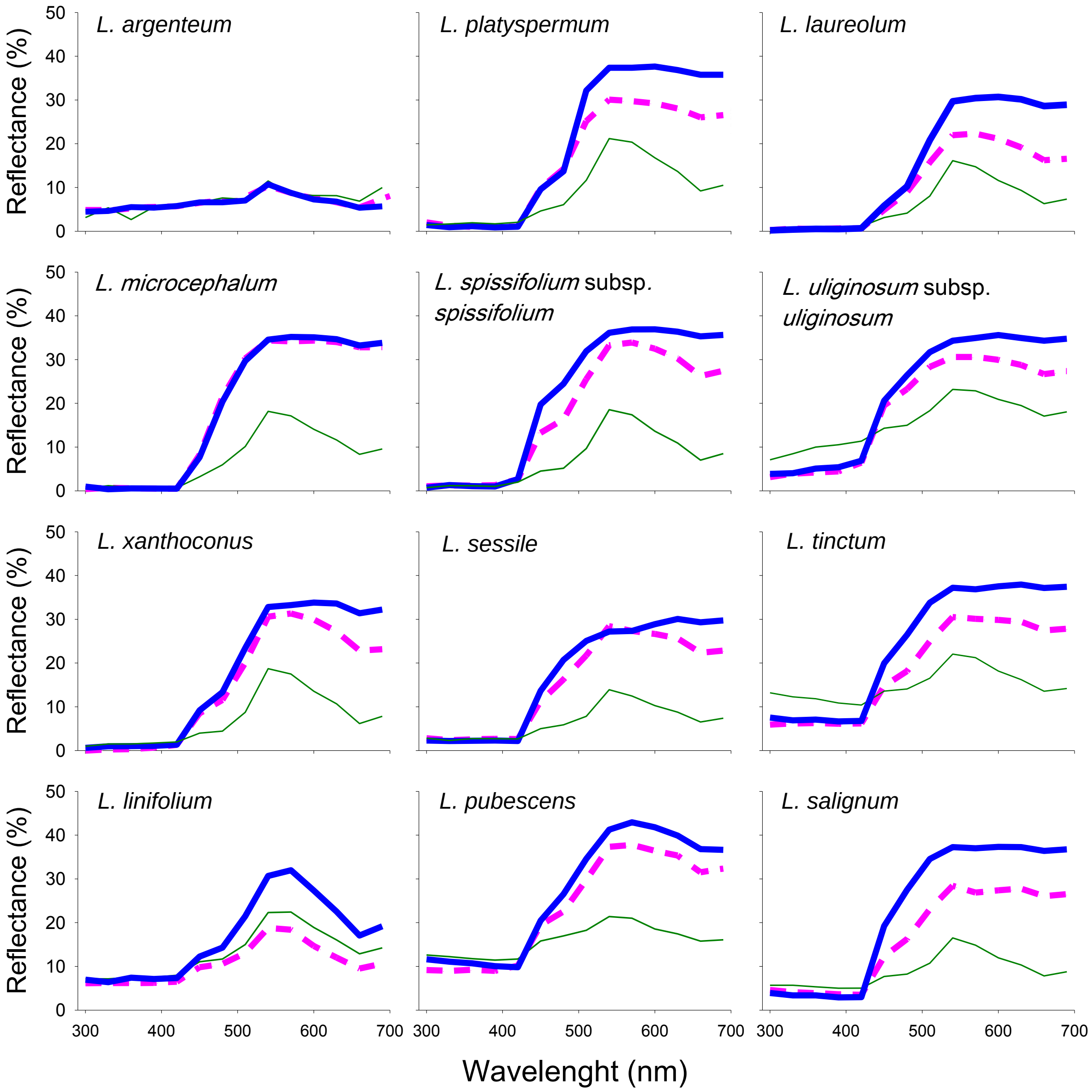

Figure S4. Average spectral reflectance of the showy leaves surrounding male (solid thick blue line) and female (dashed thick pink line) inflorescence of insect-pollinated *Leucadendron* species and green background leaves (solid thin green line) d. *n* = 8 for each of the average curves.

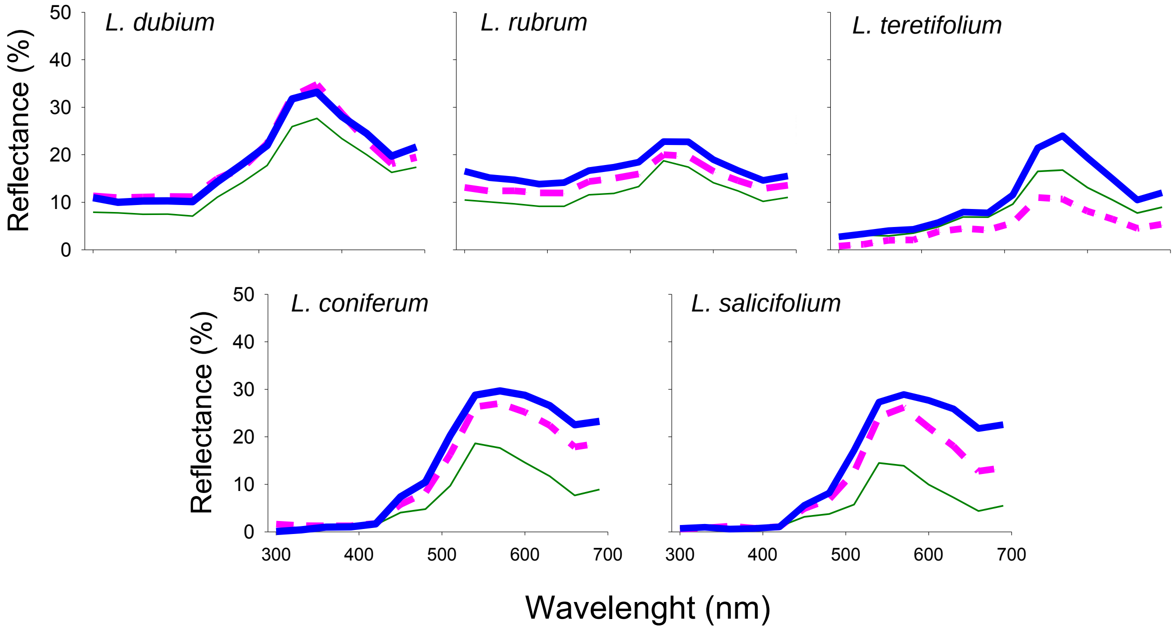

Figure S5. Average spectral reflectance of the showy leaves surrounding male (solid thick blue line) and female (dashed thick pink line) inflorescence of wind-pollinated *Leucadendron* species and green background leaves (solid thin green line). *n* = 8 for each of the average curves.

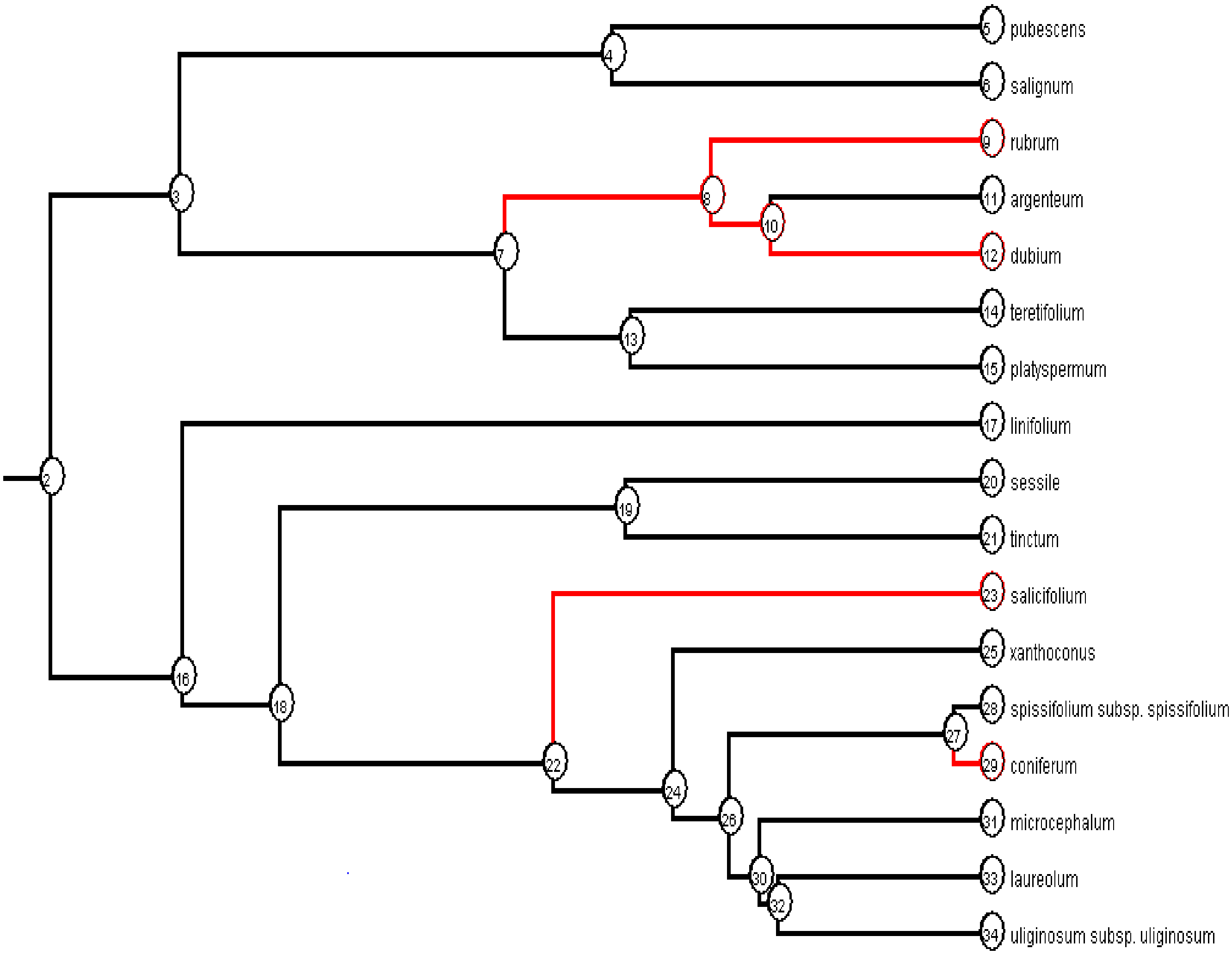

Figure S6. The node numbers related with ancestral state reconstruction of pollination system on the Bayesian maximum-clade credibility chronogram including 17 *Leucadendron* species, see Table S2 for each nodes support values. Species with red branches are wind-pollinated. Branch lengths do not represent clade age.

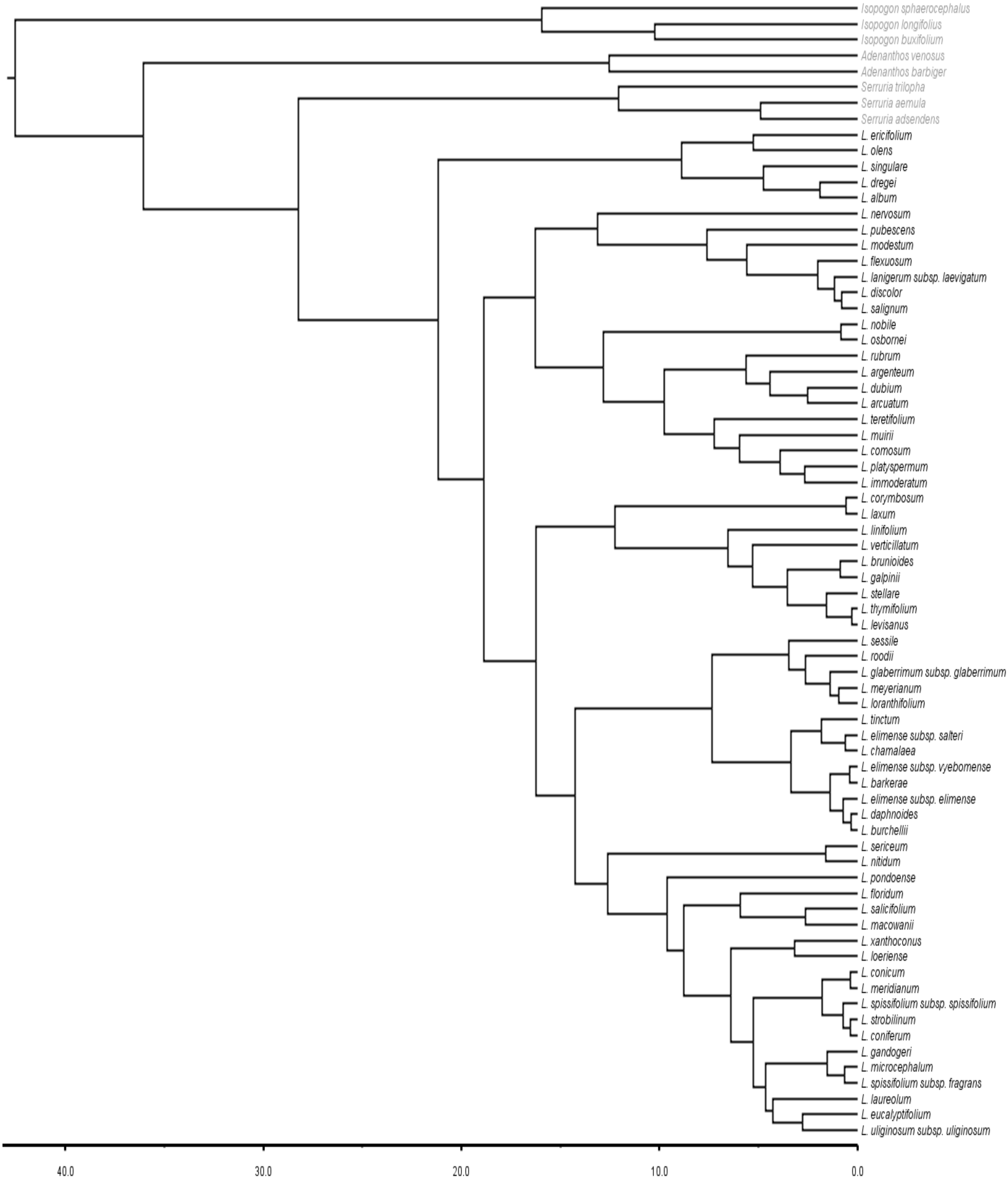

Figure S7. Dated Bayesian Maximum clade credibility tree of *Leucadendron* (as pre Hoffmann 2012).

CHAPTER 4

CONCLUDING DISCUSSION

In this thesis, I experimentally established that wind pollination occurs in at least five of 15 *Leucadendron* species examined, and from the available phylogenies can conclude that there were at least four transitions from insect to wind pollination in the genus (Chapter 2 & 3). Furthermore, by studying a wide range of traits in a phylogenetically-corrected statistical framework, I was able to identify key traits that underwent modification during these evolutionary shifts (Chapter 3). In this concluding chapter, I highlight the findings of this study and discuss the implication of these results and how they add to our current understanding of wind pollination and trait evolution in *Leucadendron*. Finally, suggestions are put forth for future research on the effects of density on individuals of wind- and insect-pollinated *Leucadendron* species, and the functional roles of traits, particularly whether scent and/or colour is a key factor in attracting pollinators, and finally suggest further work on population differentiation in *Leucadendron spissifolium*, as the various subspecies of this taxon occurring in the Western Cape and KwaZulu Natal may represent pollination ecotypes.

WIND AND INSECT POLLINATION IN *LEUCADENDRON*

The evolutionary shift from animal pollination to wind pollination signifies one of the major transitions in flowering plants (Culley et al., 2002; Friedman and Barrett, 2009). Wind pollination has evolved frequently in numerous flowering plant lineages, arising at least 65 times (Faegri and van der Pijl, 1979; Linder, 1998). Williams (1972) was the first person to suggest that within *Leucadendron* (Proteaceae), several lineages may have undergone shifts to wind pollination, based on his observations of floral traits associated with wind pollinations, such a reduction of the hypogynous scale (nectaries) and showering of pollen when branches are shaken, in several extant species. Hattingh and Giliomee (1989) experimentally confirmed wind pollination in only one species, *L. coniferum*, and insect pollination in several *Leucadendron* species. This study went a step further by comparing experimentally the potential for wind and insect pollination in a sample of 15 *Leucadendron* species chosen to be representative of as many clades as possible (Chapter 2).

Firstly, I hypothesised that experimental exclusion of insects would have a greater effect on seed production in species with traits associated with insect pollination than in those with traits associated with wind pollination. Insect exclusion experiments, which were implemented by bagging virgin budding female inflorescences with material that allowed the passage of air borne pollen, but not insects, confirmed this hypothesis, with seed set being greatly reduced in species with the insect pollination syndrome, but not in species with the wind pollination syndrome (Chapter 2). Waser et al. (1996) queried the effectiveness of using pollination syndromes in animal-pollinated plants, however for numerous plant groups there is often no doubt about the functional group of pollinators (see Fenster et al., 2004). The idea of a “wind pollination syndrome” has been relatively uncontroversial, perhaps due to the well established and often less variable traits frequently associated with wind pollination (Friedman and Barrett, 2009). This study further confirms the value for predicting wind pollination based on pollination syndromes and includes information on traits such as spectral reflectance and volatile emissions that have not traditionally been included in studies of transitions between insect and wind pollination (but see Wragg and Johnson 2011).

Secondly, I hypothesised that a greater abundance of insects would be present on insect-pollinated *Leucadendron* species. Floral visitors were sampled on female inflorescences with the tiny nitidulid beetle, *Pria cinerascens*, being found to be the main pollinator of several insect-pollinated *Leucadendron* species based on their abundance and relatively pure *Leucadendron* pollen load (Chapter 2), which confirmed similar findings by Hattingh and Giliomee (1989). Hemborg and Bond’s (2005) study on *Leucadendron xanthoconus* indicated how *P. cinerascens* depends entirely on the plants for which they pollinate in order to survive and reproduce. The male flowers provide a food source (pollen), mating and egglaying site, whereas they suggest that female flowers provide the beetles with shelter from inclement weather. Consequently, there appears to be a close specialized relationship between *P. cinerascens* and *Leucadendron*, which could be explored further. Although numerous insects visited and pollinated insect-pollinated flowers, there was no overall significant difference in insect abundance between inflorescences of insect- and wind-pollinated species. These results suggest that the patterns of insect visitation are unreliable as a measure of whether plants are insect- or wind-pollinated, and that selective exclusion experiments are required to confirm the extent to which insects contribute to pollination.

Experimental studies of wind pollination have been mainly confined to grasses (Poaceae), sedges (Cyperaceae) and trees (see reviews by Cox, 1991; Culley et al., 2002; Friedman and Barrett, 2009). My experimental studies of wind pollination in *Leucadendron* involving selective exclusion and documentation of pollen motility in a wind tunnel fill a gap in knowledge of the reproductive biology of this plant group. Importantly, this study established that wind pollination was equally effective as insect pollination (Chapter 2), which adds to the growing body of literature that shows that wind pollination, often thought of as ineffective, may be equally as effective as animal pollinators (see Cox, 1991; Midgley and Bond, 1991; Friedman and Barrett, 2009). Verification of both insect and wind pollination systems in a broad range of *Leucadendron* species was essential to create a platform for subsequent phylogenetic analysis of floral trait modifications associated with pollination system transitions in *Leucadendron* (Chapter 3).

Although a broad range of *Leucadendron* species were sampled, more species from each of the clades determined in Barker et al. (2004) should be studied, especially the probable wind-pollinated *L. ericifolia* (Williams, 1972). I mainly focused on species close to the Cape Peninsula, which should be extended to *Leucadendron* species in more remote locations in order to have a complete picture of pollination systems in the genus. Furthermore, even though pollination by *P. cinerascens* and monkey beetles was determined for several species, for species such as *L. pubescens* and *L. linifolium* the main pollinator eluded me.

FLORAL TRAIT SHIFTS

For numerous angiosperm lineages, phylogenetic studies indicate that speciation is related to pollination system shifts and the linked shifts in floral traits (Whittall and Hodges, 2007; Valente et al., 2012; Van der Niet and Johnson, 2012; Forest et al., 2014). The evolutionary shift to wind pollination is commonly associated with a specific suite of floral morphological traits, such as small inconspicuous flowers with fewer (often single) ovules, reduced or absent perianth, loss of nectaries and light, dry, smooth, small pollen grains (Faegri and van der Pijl, 1979; Culley et al., 2002; Friedman and Barrett, 2008, 2009; Wragg and Johnson, 2011). However, phylogenetic analyses suggest wind pollination evolves more frequently in clades that have morphological traits facilitating the transition, so that only a few trait modifications, notably dry pollen and small flowers, are necessary for the transition to wind pollination (Linder, 1998; Friedman and Barrett, 2009; Wragg and Johnson, 2011).

A key aim of this study was to identify traits which were modified during shifts from insect to wind pollination. I hypothesised that several floral trait modifications associated with wind pollination would occur during the shift from insect to wind pollination in *Leucadendron* species. I identified production of more abundant amounts of smaller pollen grains that are highly motile, reduced floral scent emission, less colourful "showy" leaves surrounding inflorescences, greater inflorescence density and generally a greater degree of sexual dimorphism as being traits which likely evolved during the shift to wind pollination from insect-pollinated ancestors. Wragg and Johnson (2011) determined that the evolution of floral scent emission, showy floral colour and low motile pollen is associated with the shift from wind to insect pollination in sedges. Importantly, in this study, the likelihood that the modifications in these traits were associated with the insect to wind pollination shift was statistically significant in analyses that take phylogenetic relatedness among species into account (Chapter 3). Similar approaches have been used in recent studies of floral nectary evolution (Hobbhahn et al., 2013) and variation in sex-ratios among dioecious plants (Field et al., 2012).

FUTURE STUDIES

A critical issue is to understand the ecological circumstances that favour shifts between insect and wind pollination. One possibility is that wind pollination is favoured when levels of insect pollination are limiting to plant fitness. However, wind pollination may only be effective in dense populations. *Leucadendron* offers an ideal system to test the effects of plant aggregation on the efficiency of insect versus wind pollination with the prediction being that wind pollination will be less effective than insect pollination in sparse populations. In other words, wind-pollinated species should be more vulnerable to density Allee effects (Davis et al., 2004). As the species are dioecious, it could be tested how the distance from male plants affects fecundity of female plants, for both insect- and wind-pollinated species.

Another key question for future studies is to identify the functional roles for traits that were modified during insect to wind pollination shifts. For example, this study demonstrated that insect-pollinated species produce numerous floral scent compounds compared to wind-pollinated species, including esters which are known to attract beetle pollinators. Fermented-fruit and yeasty scents are suggested to play a role in attracting beetle pollinators of Annonaceae (Goodrich et al., 2006; Gottsberger et al., 2011). However, it is unknown what role scent plays in attracting pollinators to insect-pollinated *Leucadendron* inflorescences. Therefore, GC-EAD (gas chromatography-electroantennographic detection) could be conducted to determine which of the numerous floral volatiles emitted by inflorescences of insect-pollinated *Leucadendron* species produce a physiological antennal response by pollinators, in particular *Pria cinerascens* the main pollinator of several species. Following this screening of compounds inducing a response, scent bioassays such as olfactometer apparatus and/or field trapping dose-dependent responses can be used to test the beetle's attraction to these specific compounds or a blend of compounds (Salzmann et al., 2007; Peakall et al., 2010). These tests would not only shed light on the general role of scent in attracting *P. cinerascens* but also help to identify specific compounds that may mediate the interactions between *Leucadendron* species and *P. cinerascens*.

Another floral attractant that should be investigated further is colour. The yellow colouration of the leaves surrounding the inflorescences of insect-pollinated species differed more from the green background leaves than did those of wind-pollinated species. Therefore, field experiments using yellow coloured apparatus, such as pan traps or painted cardboard, could be used to investigate whether the yellow colouration of these leaves acts as an attractant to beetle pollinators For instance, using various colour models, Johnson and Midgley (2001) determined that colour rather than floral patterns were the primary influence alighting behaviour of monkey beetles. Furthermore, a combination of scent and colour field experiments could determine whether only scent, only colour or a combination of both are important in attracting pollinators. Steenhuisen et al. (2013) determined in field experiments using scent and colour that cetoniine beetles were highly attracted to a combination of linalool (scent compound) and yellow traps rather than other combinations using green traps or paraffin. Finally, investigating differences or similarities between the scent and colour attractants of male and female inflorescences could yield interesting insight into what attracts pollinators to female inflorescences, given they have no apparent reward. For instance, sexually dimorphic plants are suggested to differ in floral scent, given that floral organs can vary in compounds emitted (Dobson and Bergstrom 2000; Ashman et al., 2005). However, Ashman's (2009) review on patterns of floral scent in sexually dimorphic plants found that in species with rewardless females, sexual dimorphism in floral scent was generally less common (i.e. female plants might be mimicking male plants' scent in order to attract pollinators).

Finally, an interesting aspect to research on *Leucadendron* is the spread of the *Leucadendron spissifolium* lineage into KwaZulu Natal. *Leucadendron spissifolium* has five sub species, three occur in the Western Cape, i.e. *L. spissifolium* (Salisb. ex Knight) I. Williams subsp. *spissifolium*, *L. spissifolium* subsp. *fragrans* I. Williams, *L. spissifolium* subsp. *phillipsii* (Hutch.) I. Williams, while two subspecies occur in KwaZulu Natal, i.e. *L. spissifolium* subsp. *natalense* (Thode & Gilg) I. Williams and *L. spissifolium* subsp. *oribinum* I. Williams. These two KwaZulu Natal subspecies along with *L. pondoense* A.W.

van Wyk are the only *Leucadendron* species occurring outside of the Western Cape in South Africa. Consequently, investigating the differentiation in the pollination system of those subspecies occurring in the Western Cape compared to those in KwaZulu-Natal could yield interesting results. A key issue to be determined is whether the main pollinator of *Leucadendron*, the nitidulid beetle *P. cinerascens*, is found pollinating these species in KwaZulu-Natal. Since, I found that monkey beetles were the main pollinators of *L. spissifolium* subsp. *spissifolium* (Chapter 2), and given that monkey beetles have been observed visiting *L. spissifolium* subsp. *oribinum* (S.D. Johnson pers. obs.), monkey beetle pollination might hold the key to the successful expansion of this linage outside the Cape Floristic Region.

CONCLUSION

This study confirms that there were at least five shifts from insect to wind pollination in *Leucadendron* and identifies traits that were modified during these shifts. Further research should be aimed at 1) determining the effects of plant spacing on fitness of individuals of wind- and insect-pollinated species, 2) identifying the functional significance of traits, particularly scent and colour, for attracting insect pollinators, and 3) examining how shifts between different insect groups may have promoted diversification among geographically separated populations of insect-pollinated species.

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